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SANOCRYSIN IN THE TREATMENT OF TUBERCULOSIS IN THE RABBIT.

COL. S. LYLE CUMMINS, C.B., C.M.G., M.D., A.M.S. (RET. PAY),
David Davies Professor of Tuberculosis;

ASSISTED BY MISS C. M. ACLAND.

From the Central Tuberculosis Laboratory, Medical School, Cardiff.

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THE experiments to be described were undertaken in the hope of throwing light upon the following points:

(1) Does sanocrysin treatment favourably modify the course of experimental tuberculosis in the rabbit?

(2) Does sanocrysin lead to "tuberculin shock" in the tuberculous rabbit, and if so, can this shock be prevented by Möllgaard's anti-tuberculous serum?

(3) To what extent do quantitative considerations, *i.e.* differences in the infecting bacillary dose and in the virulence of the infecting strain of tubercle bacillus, enter into the action, if any, of sanocrysin in tuberculous rabbits?

Experiment 1.

Three animals, Rabbits 29, 30 and 31, already infected with tuberculosis, were kindly placed at my disposal by Dr. Tytler. Each of these rabbits had received, on 22 . 1 . 25, a subcutaneous inoculation of an emulsion of a virulent bovine culture, estimated to contain 500,000 tubercle bacilli.

By 2 . 3 . 25 all had started to lose weight, and were, therefore, assumed to be heavily infected.

On that date each of them was given 0·1 gm. of sanocrysin intravenously; and Rabbit 31 was also given an intramuscular injection of 2 c.c. of anti-tuberculous serum.

On 3 . 3 . 25 none of the animals exhibited any sign of "shock," save that the breathing was much more rapid in Rabbits 29 and 30 than in Rabbit 31. Rabbits 30 and 31 were now given 2 c.c. of serum.

On 4 . 3 . 25 all three animals were still free from any symptom of shock, though the breathing continued more rapid in Rabbits 29 and 30 than in Rabbit 31.

Further doses of sanocrysin were given to all three animals as follows:

9 . 3 . 25 . . sanocrysin 0·05 gm.
16 . 3 . 25 . . sanocrysin 0·025 gm.

On each of these dates Rabbit 31 was given, at the same time, 2 c.c. of serum, the other animals being left without serum treatment. In none were symptoms of shock observed.

In Rabbit 29 the weight started to fall rapidly on 29 . 4 . 25, and the animal died of generalized tuberculosis on 20 . 5 . 25. Rabbit 31 started to lose weight rapidly on 6 . 5 . 25, and died of tuberculosis on 21 . 5 . 25.

On 21 . 5 . 25, Rabbit 30, too, appeared to be dying, having rapidly lost weight from 13 . 5 . 25 onwards. An intramuscular injection of 4 c.c. of anti-tuberculous serum was now given. On 22 . 5 . 25 the animal appeared better and was eating, but the breathing was very rapid. On 25 . 5 . 25 the animal was still alive, but appeared greatly wasted and very ill and dyspnœic. An intravenous injection of 0·05 gm. of sanocrysin was given along with 2 c.c. of serum intramuscularly. The animal died half an hour later.

In all three animals the lungs were infiltrated with caseous tuberculosis and the kidneys showed fairly extensive disease.

Results of Experiment 1.—The absence of " untreated controls " restricted the utility of this experiment, but it sufficed to show that doses of sanocrysin amounting to 0·175 gm. had failed to arrest the progress of experimental tuberculosis following the injection of a virulent bovine strain. As there had been no definite evidence of " shock " after the injections of sanocrysin, it was not possible to draw any conclusion as to the utility of the serum.

Experiment 2.

Four animals were used. Of these, Rabbits 17 and 18 had been immunized against *Staphylococcus aureus* a year before in the course of experiments on thermo-stable opsonins. Rabbits 20 and 21 had had a subcutaneous inoculation of *human* tubercle bacilli over a year previously, in the course of another experiment. Both animals were now in good condition and up to their normal weight.

For this experiment a strain of bovine tubercle bacilli, obtained some years previously from the Lister Institute, and which had proved, in a previous experiment, to be of *low* virulence, was used.

An emulsion of a four-weeks' culture on egg medium was prepared of an opacity corresponding to " No. 5 " on Brown's (1919) scale.*

Of this emulsion, an intravenous inoculation of 0·5 c.c. was given to each of the four rabbits on 26 . 3 . 25. The animals were observed for six weeks and, at the end of that time, still remained in good condition, but in view of the large intravenous dose given they were now assumed to be infected.

On 6 . 5 . 25 Rabbits 18 and 20 received each 0·1 gm. of sanocrysin

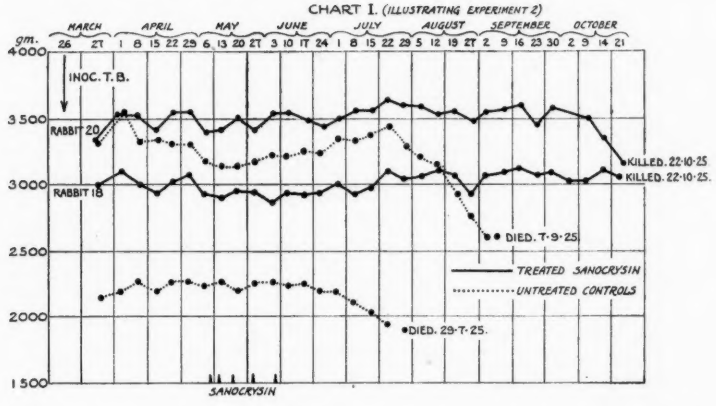
* This " opacity " has been found by us to correspond to a " count " of approximately 5000 million tubercle bacilli per 1 c.c. of the emulsion.

intravenously, and this dose was repeated weekly up to and including 3 . 6 . 25. In the case of Rabbit 18, an intramuscular dose of 4 c.c. anti-tuberculous serum was given with the first two doses of sanocrysin but not with the last three.

Möllgaard (1924) places the "*dosis tolerata*" for the intravenous inoculation of sanocrysin in Rabbits at 4 cgm. per kilo of body-weight. It is to be noted that Rabbits 18 and 20 tolerated five successive doses of about 3·3 cgm. per kilo at 7-day intervals without any obvious ill-effects or symptoms of "shock." It is to be regretted that circumstances prevented the collection of urine for analysis; but neither animal had shown any significant loss of weight or condition during the five weeks of treatment, and both continued to feed well.

Rabbits 17 and 21 were left untreated to serve as controls.

CHART I. (ILLUSTRATING EXPERIMENT 2)



Results of Experiment 2.—(1) Both the "untreated controls" died within less than five months of infection from extensive tuberculosis, affecting chiefly the lungs and kidneys. Rabbit 21 died on 29 . 7 . 25 and Rabbit 17 on 7 . 9 . 25.

(2) Both the rabbits treated with sanocrysin survived in good condition until it was decided to investigate the extent of their infection if any.

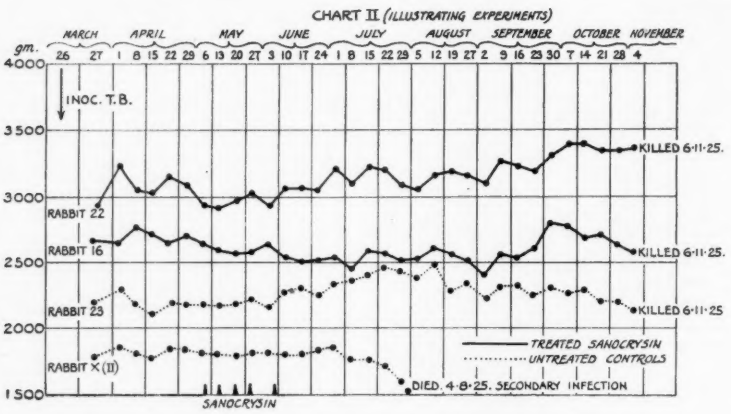
Rabbits 18 and 20 were *killed* on 22 . 10 . 25. Although there was definite pulmonary tuberculosis in both, this was of a chronic type, the caseous areas being isolated and surrounded by relatively healthy lung. In Rabbit 18, especially, the areas of disease were so well limited by reparative processes that the animal might well have gone on to complete recovery; but it is probable that Rabbit 20 would have, before long, died of the disease.

The weight changes in these four rabbits, with dates of treatment, etc., are illustrated in Chart I. The macroscopic appearances of the lungs are shown in Plate I. The histological characters of the lung lesions are illustrated in Plate II. A careful examination of the sections shows that, while the tuberculous process has, in the lungs of the untreated animals, Rabbits 17 and 21, led to the formation of many caseo-necrotic areas in which

the cellular elements have almost disappeared, leaving nothing but granular *débris* and a few pyknotic nuclei, the lesions in the lungs of Rabbits 18 and 20, though extensive in the portions chosen for section, consist for the most part of vascular granulation-tissue in which the nuclei are intact and tending to proliferation. The presence of numerous areas of well-aërated lung-tissue indicates that the defensive process has met with considerable success. The lungs of Rabbits 17 and 21 have almost entirely lost their alveolar structure and most of the blood-vessels have disappeared. In the lungs of Rabbits 18 and 20, however, there are, in addition to emphysematous portions, areas of almost normal aëration with intact alveoli while blood-vessels are numerous through the sections.

Experiment 3.

This experiment was a replica of Experiment 2, save that the "infecting dose" consisted of an emulsion of "T.B. Richards" (human) of opacity



corresponding to No. 5 of Brown's scale, and that a larger dose, 0·75 c.c., was given. The intravenous infecting inoculations were given to all four animals on 26 . 3 . 25.

Rabbit 16 had previously been immunized against *Staphylococcus aureus* as in the case of Rabbits 17 and 18. Rabbit X (11) had received a small dose of human tubercle bacilli into the tissues of the left ear in January, 1923, for the study of a restricted tissue lesion; while Rabbits 22 and 23 had both sustained a subcutaneous inoculation of human tubercle bacilli early in 1924, at the same time as Rabbits 20 and 21.

As in Experiment 2, an interval of 6 weeks was allowed to elapse, and then 2 of the rabbits, Nos. 16 and 22, were placed on sanocrysin treatment, 5 doses of 0·1 gm. being given to each at intervals of a week between 6 . 5 . 25 and 3 . 6 . 25. Rabbits X (11) and 23 were kept as "untreated controls." Rabbit X (11) died on 4 . 8 . 25 from an intercurrent disease and, at post mortem, was found to have numerous small tubercles throughout the lungs which had not

yet developed to the markedly caseous stage. The remaining three animals remained in apparently good health during September and October, and were killed on 6 . 11 . 25.

Results of Experiment 3.—The lungs of Rabbit 23, the surviving "untreated control," were found to be heavily infected, while both kidneys showed numerous small tubercles.

The lungs of the two animals treated with sanocrysin, Rabbits 16 and 22, presented a marked contrast to those of Rabbit 23. In the case of Rabbit 16 the lungs appeared to be normal, and those of Rabbit 22 were also almost free from signs of disease, although some small grey flecks, possibly healed tubercles, were visible at the bases. The kidneys of Rabbit 16 showed a couple of bluish-grey patches representing healed tuberculous lesions, while one small caseous point was seen under the capsule of the right kidney in Rabbit 22.

The macroscopic appearances of the lungs of Rabbits 16, 22 and 23 are illustrated in Plate III, and their histological characters in Plate IV. In the latter it will be seen that, while the lungs of Rabbit 23 are full of necrotic areas and almost without any alveolar structure, those of Rabbit 22 are normal save for some congestion, and show no trace of tuberculosis. Chart II shows details of the experiments.

Experiment 4.

This was a repetition of Experiment 2, but with a larger dose of bovine bacilli.

Four animals were used, Rabbits 6(*a*), 9, 38 and 39. All four had received a conjunctival instillation of 0·02 c.c. of bovine bacillary emulsion five months before, but none had yet shown definite signs of infection.

The infecting dose was given to each of them on 25 . 9 . 25, and consisted of an intravenous inoculation of 0·5 c.c. of a *No. 10* (Brown's scale) emulsion of bovine bacilli.

Rabbit 38 died on 16 . 10 . 25, showing marked early tuberculosis of both lungs. Death was probably due to some secondary bacterial infection, the nature of which was not determined.

On 20 . 10 . 25 all the surviving animals had commenced to lose weight.

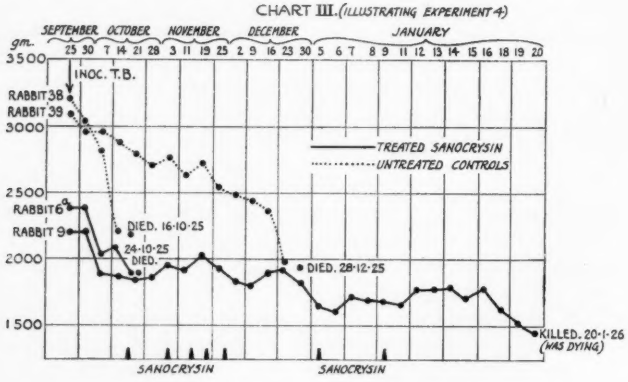
On that date Rabbits 6(*a*) and 9 were each given 0·1 gm. of sanocrysin as an intravenous inoculation. Rabbit 6(*a*) died on 24 . 10 . 25, death being, perhaps, accelerated by the sanocrysin dose. The lungs showed a degree of tuberculosis comparable to that seen in Rabbit 38.

There remained Rabbit 9, and the "untreated control," Rabbit 39.

Rabbit 9 received five doses, each of 0·1 gm. sanocrysin, between 20 . 10 . 25 and 1 . 12 . 25. Rabbit 39, the untreated control, died on 28 . 12 . 25. The lungs and kidneys were found to be extensively infiltrated with caseous tubercles. Rabbit 9 began to lose weight on 30 . 12 . 25 and was very poorly on 5 . 1 . 26, when a dose of 0·05 gm. of sanocrysin was given along with 3 c.c. of serum. A further dose of 0·1 gm. of sanocrysin was given on 11 . 1 . 26, and was well tolerated, weight being regained and maintained up to 16 . 1 . 26. A rapid fall in weight, however, took place from the 18th to the 20th January, when the animal seemed unlikely to survive for long and was, therefore, killed.

Results of Experiment 4.—The lungs of the treated animal, Rabbit 9, though extensively diseased and infiltrated with tuberculosis, showed to the naked eye a very definite tendency to fibrosis. It was in the kidneys, however, that the sharpest contrast to Control Rabbit No. 39 was seen. The left kidney had obviously been extensively affected with tuberculosis, but the lesions were found at post-portem to have been converted, almost entirely, into fibrous tissue which had contracted between islands of healthy tissue. In the right kidney there was a large fungating tuberculous mass of caseo-fibrous consistence rising, like a new growth, from the surface of the organ and surrounded, at the base, by a zone of fibrous tissue. The rest of the organ was riddled with the fibrous remains of healed tubercles similar to those seen in the left kidney. Sections of the kidneys of Rabbits 9 (treated)

CHART III. (ILLUSTRATING EXPERIMENT 4)



and 39 (untreated) are shown in Plate V. Chart III shows details of the experiment.

DISCUSSION.

These observations prove that the value of sanocrysin depends upon the following factors: the extent of the tuberculosis at the commencement of treatment, the virulence and dose of the infecting strain, and the resistance of the animal concerned.

It is recognized that these three factors are closely related and inter-dependent, but each can, to some extent, be controlled under experimental conditions; the extent of the disease being determined by the dose, the virulence, and the time allowed to elapse after infection; while the resistance can be made to differ according to the species of animal selected as well as by the size of the infecting dose and the bacterial type.

Experiment 1 is unsatisfactory because no untreated "controls" were included, and because the dose of sanocrysin was, in all probability, too small to be effective.

In Experiments 4, 2 and 3, however, where the "dose-virulence" factor was in a descending scale, the success of the sanocrysin was found to vary

inversely with the magnitude of the infection and the extent of the disease at the time of the inception of the treatment.

In Experiment 4, where the largest infecting dose was given, the three surviving animals had commenced to lose weight when the treatment was started, indicating that the disease was already extensive—an assumption which was confirmed by the condition of the lungs in Rabbit 38, which died of a secondary infection four days before the others were placed on "sanocrysin," and in Rabbit 6(*a*), which died after the first dose of sanocrysin.

Here, Rabbit 9, the treated animal, only outlived the untreated control by a few weeks, and though the favourable influence of sanocrysin was clearly seen, the lung infection was, nevertheless, very extensive and incompatible with long survival.

In Experiment 2 the fact that all the animals were still up to weight and in good condition at the start of treatment shows that the disease was not, at that time, very far advanced.

For the degree of infection produced by the infecting dose, which was much smaller than in Experiment 4, the sanocrysin treatment was of considerable value, as shown by the fact that the treated animals outlived the "untreated controls" for eleven weeks and six weeks respectively, and were in such good condition when *killed* that a considerable survival period was to be expected, or even, perhaps, recovery in the case of Rabbit 18.

In Experiment 3, in which the animals were infected with a large dose of "human" tubercle bacilli, against which the rabbit possesses a high degree of natural resistance, the disease was definitely arrested by the sanocrysin treatment while it *progressed* in the surviving "untreated controls," as is evident from the far more advanced disease noted in Rabbit 23 when killed on 6.11.25 than was found in Rabbit X (11) dying of a secondary infection three months earlier.

While these experiments throw no light on the exact mode of action of sanocrysin, they confirm the animal experiments of Möllgaard in showing that this preparation leads, in some way, to the destruction of many of the bacilli in tuberculous foci. It is significant that the action of sanocrysin upon lesions in the kidneys, the organs in which it is found to attain its highest concentration (Frandsen, 1925, T. R. Elliott, 1926, McCluskey and Eichelberger, 1925), was still more marked than upon lesions in the lungs.

CONCLUSIONS.

(1) Sanocrysin, used in appropriate doses, and at an early stage in the disease, has a markedly favourable action upon the course of experimental tuberculosis in the rabbit.

(2) As no definite evidence of the production of "shock" was forthcoming in these experiments, it was impossible to evaluate the results of Möllgaard's anti-tuberculous serum.

(3) The favourable effects of sanocrysin upon the tuberculosis of the rabbit vary inversely with the virulence of the infecting bacillus and the size of the infecting dose. The conclusion is important, because it has a bearing upon the selection of "human" cases for treatment.

This investigation was rendered possible by the provision of sanocrysin and serum by the Medical Research Council, for which grateful acknowledgment is made.

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DESCRIPTION OF PLATES.

PLATE I.

Figs. 1 *and* 2 *illustrate Experiment* 2.

Fig. 1 is a photograph of the lungs of Rabbit 18 infected with bovine tubercle bacilli and subsequently treated with sanocrysin. Fig. 2 is a photograph of the lungs of Rabbit 17, an "untreated control."

PLATE II.

Figs. 1 *and* 2 *illustrate Experiment* 2.

Fig. 1 is a microphotograph of a section of the lung of Rabbit 18 treated with sanocrysin. × 300. Fig. 2 is a microphotograph of a section of the lung of Rabbit 17, an untreated control. × 300.

PLATE III.

Figs. 1, 2 *and* 3 *illustrate Experiment* 3.

Figs. 1 and 3 are photographs of the lungs of Rabbits 22 and 16 treated with sanocrysin after infection with human tubercle bacilli. Fig. 2 is a photograph of the lungs of Rabbit 23, an "untreated control" infected with human tubercle bacilli.

PLATE IV.

Figs. 3 *and* 4 *illustrate Experiment* 3.

Fig 3 is a microphotograph of the lung of Rabbit 22, treated with sanocrysin after infection with human tubercle bacilli. × 300. Fig. 4 is a microphotograph of the lung of Rabbit 23, an "untreated control" infected with human tubercle bacilli. × 300.

PLATE V.

Figs. 5 *and* 6.

Fig. 5 is a microphotograph of a section of the left kidney of Rabbit 9, treated with sanocrysin after massive infection with bovine tubercle bacilli. It illustrates a healing tuberculous lesion, the contraction of fibrous tissue drawing the surface downwards into a "dimple." × 300. Fig. 6 is a microphotograph of a section of the left kidney of Rabbit 39, an untreated control, dying after massive infection with bovine tubercle bacilli. It shows an area of caseo-necrotic tuberculosis in the cortex. × 300.

FIG. 1.

FIG. 2.

Plate I.

Cummins.

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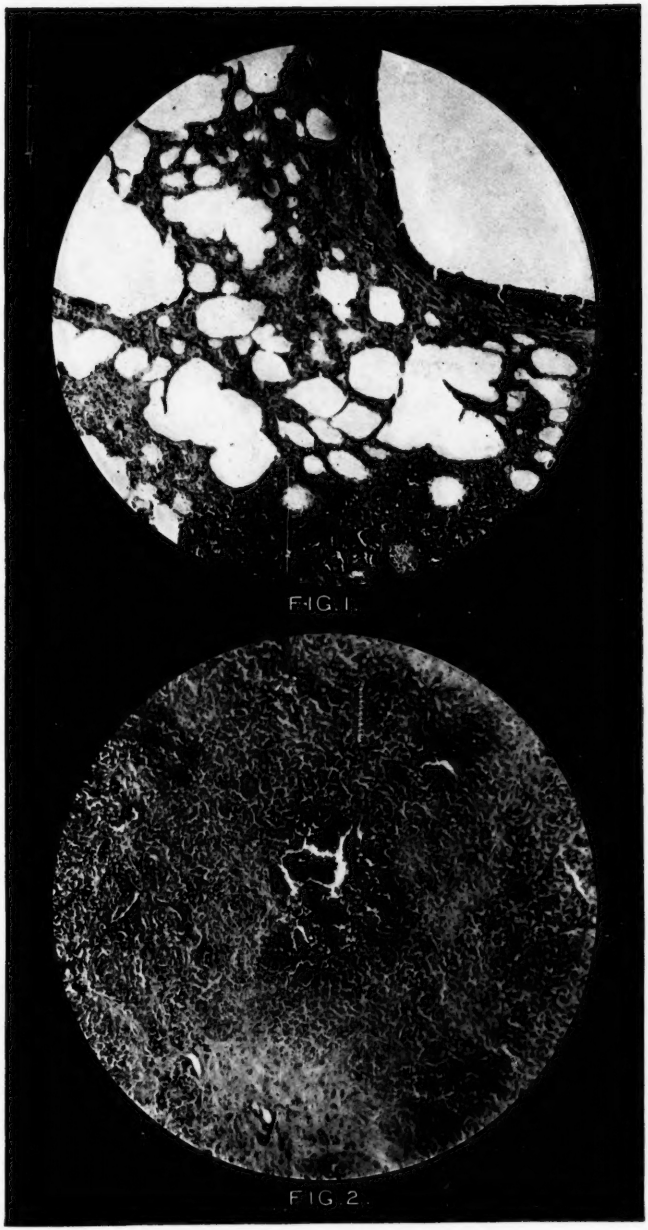

FIG. 1.

FIG. 2.

Plate II.

Cummins.

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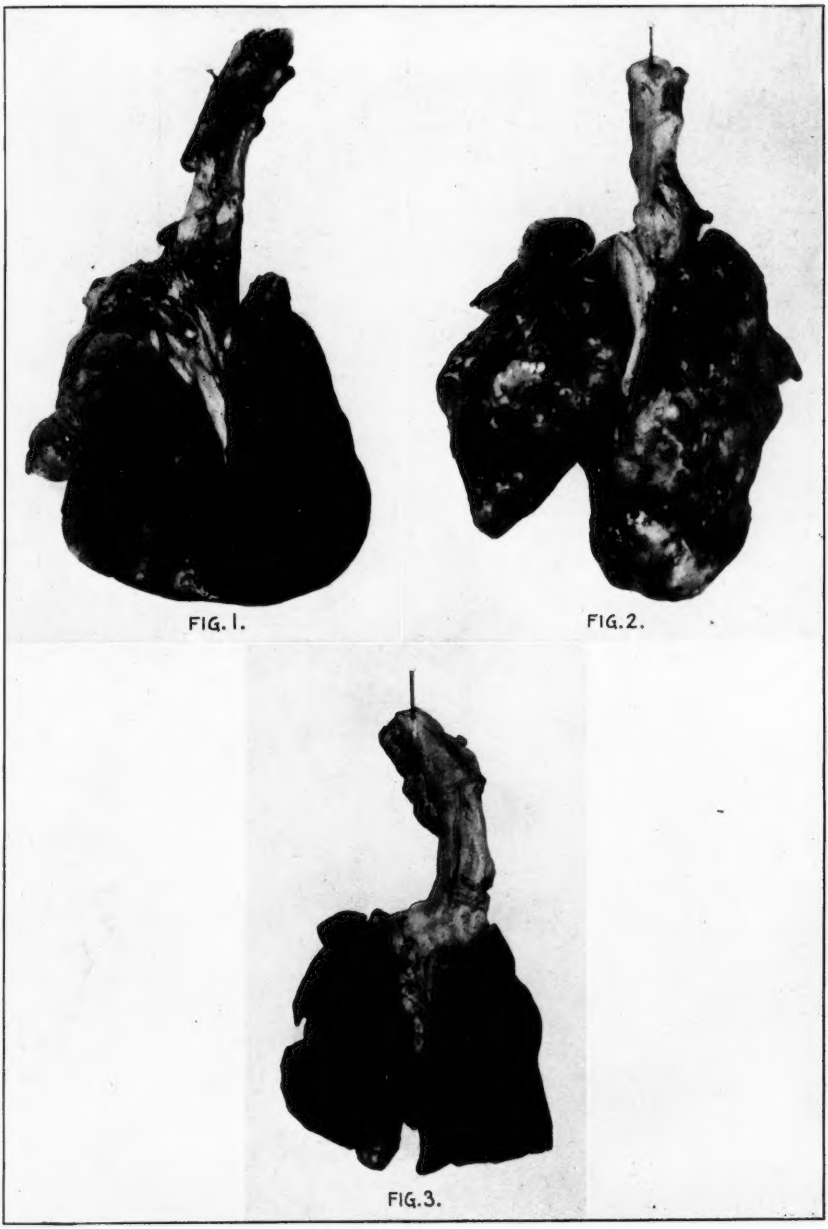

FIG. 1.

FIG. 2.

FIG. 3.

Plate III.

Cummins.

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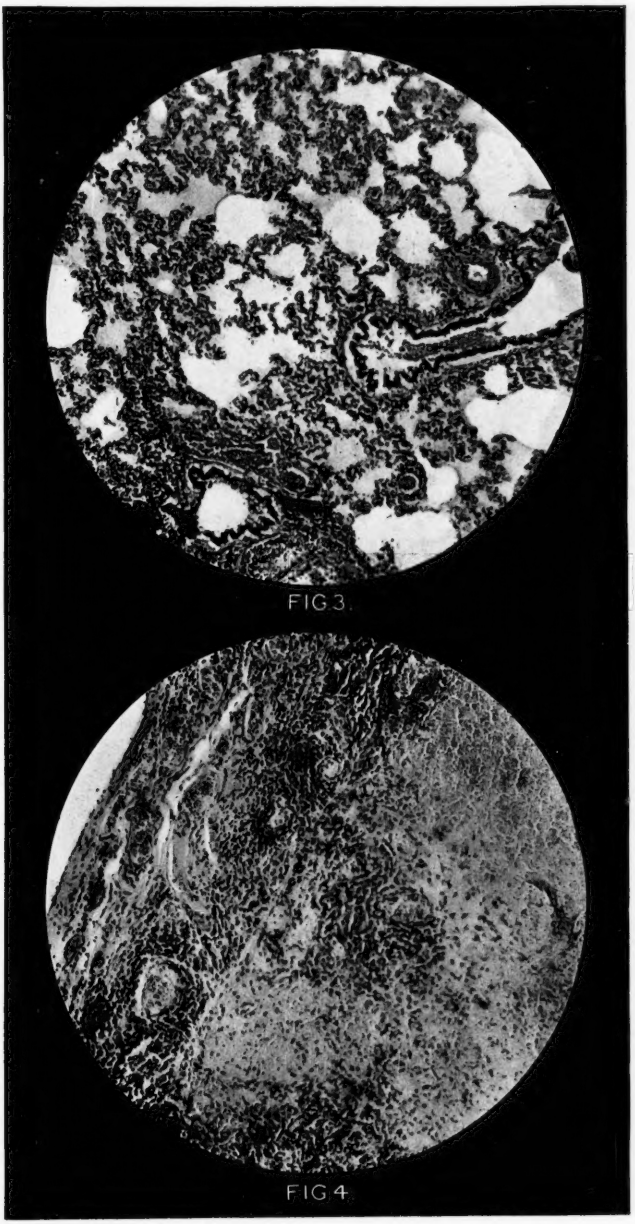

FIG 3.

FIG 4.

Plate IV.

Cummins.

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FIG. 5.

FIG. 6.

Plate V.

Cummins.

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THE ANTIGENIC PROPERTIES OF PRECIPITATES PRODUCED BY THE INTERACTION OF DIPHTHERIA TOXIN AND ANTITOXIN.—PART 2.

PERCIVAL HARTLEY.

From the National Institute for Medical Research, Hampstead, London.

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In an earlier paper (Hartley, 1925) it has been shown that the precipitates produced when diphtheria toxin and antitoxin are mixed together in certain proportions are antigenic. These precipitates, after thorough washing with normal saline, contained less than 1 per cent. of the nitrogen compounds present in the original mixtures from which they separated, and all the preparations studied were innocuous to guinea-pigs even in large doses. It was also shown that the antigenic value of such a precipitate varied with the nature of the toxin-antitoxin mixture from which it separated; if the antitoxin was in large excess the antigenic value was low, and the most active preparation was obtained from a mixture which was slightly toxic for guinea-pigs.

The results of these earlier experiments suggested that the antigenic value of precipitates prepared from mixtures which are more toxic for guinea-pigs would be greater than that prepared from mixtures which are atoxic or of only feeble toxicity. Some difficulty was experienced in preparing such precipitate, and this, it is believed, was due to the fact that old, mature toxins were used. This difficulty has now been overcome by using freshly-prepared, high-grade toxins. When gradually decreasing volumes of diphtheria antitoxin are added to constant volumes of such fresh toxins precipitation occurs in certain of the mixtures. If toxin is in large excess precipitation does not occur, while in mixtures which are nearly neutral precipitation occurs readily. In between these two groups are mixtures which, on the one hand, are lethal for guinea-pigs in small doses and, on the other hand, undergo precipitation. These mixtures first become opalescent, and after a few days precipitation begins. The precipitates are very fine and sedimentation is slow. The amount of precipitate obtained is less than that which separates from mixtures which are more nearly neutral. Further, these precipitates, after thorough washing with normal saline, are toxic for guinea-pigs, and in this respect they differ from those prepared and described in the earlier paper referred to above.

In the series of experiments described below the same toxin and antitoxin were used throughout, and the following points have been investigated. The antigenic properties of the precipitates prepared from (*a*) an over-neutralized, (*b*) a neutral, and (*c*) an under-neutralized mixture of the toxin and antitoxin have been compared; the antigenic properties of the precipitate from a

toxin-antitoxin mixture have been compared with those of the whole mixture and also with the fluid remaining after removal of the precipitate; the antitoxin production following the injection of varying quantities of the same toxin-antitoxin precipitate has been investigated; the effect of repeated washing of a toxin-antitoxin precipitate on the nitrogen content of the preparation and on its antigenic activity has been studied; and the effect of the route of administration of the washed precipitate on antitoxin production has also been investigated.

METHODS.

1. *Preparation of Reagents.*

(*a*) *Diphtheria toxin.*—The toxin used was prepared by the method of Hartley and Hartley (1922), Difco proteose peptone being incorporated in the medium. The organism was grown for seven days at 36° C., and then filtered, toluol being added as preservative.

Tests carried out at once on the fresh toxin gave the following results:

(i) The minimum lethal dose was between 0·002 c.cm. and 0·0025 c.cm.

(ii) The L_0 dose was 0·10 c.cm., or very nearly so (see Table I).

(iii) The L_+ dose was very nearly 0·13 c.cm. Animals receiving this dose of toxin mixed with 1 unit of antitoxin were found dead on the morning of the fourth day, and none survived for longer than 4 days.

(*b*) *Diphtheria antitoxin.*—The sample used contained no preservative and was stated to contain approximately 500 units per c.cm. It was kindly supplied by Dr. O'Brien, of the Wellcome Physiological Research Laboratories. It was tested before use and found to contain the units stated. (4th day death.)

(*c*) The following mixtures of toxin and antitoxin were prepared, and the precipitates which separated from them were washed and their antigenic properties investigated.

Mixture 8.—100 c.cm. of toxin + 2·5 c.cm. of antitoxin. This mixture corresponds to the one which, in the Ramon flocculation test, precipitated first in point of time. 20 c.cm. of the mixture were pipetted off and, after standing for 1 hour, 2 c.cm. doses were injected into guinea-pigs. The remaining 80 c.cm. were allowed to stand at room temperature in the dark for four days. The clear supernatant fluid was siphoned off and the sedimented precipitate was washed five times with normal saline. The washed precipitate was finally emulsified in 8 c.cm. of normal saline.

0·1 c.cm. of this emulsion contains an amount of precipitate equivalent to that which separates from 1 c.cm. of original mixture.

Mixture 9.—500 c.cm. of toxin + 10 c.cm. of antitoxin.

Assuming that the values determined for the toxin and antitoxin used are correct, this mixture is exactly neutral, *i.e.* to 5000 L_0 doses of toxin 5000 units of antitoxin have been added. 20 c.cm. of the mixture were removed, and after standing for 1 hour, guinea-pigs were injected with 2 c.cm., 1 c.cm. and 0·1 c.cm. doses. The remaining 480 c.cm. were allowed to stand for 4 days, and the supernatant fluid was siphoned off; this fluid was perfectly clear, but in order to ensure the removal of all particles, it was filtered twice through double filter-paper. The antigenic value of this filtered supernatant fluid was investigated. The precipitate was then washed. After 2 washings the precipitate was emulsified in saline and the volume made up to 48 c.cm. 8 c.cm. were withdrawn and retained for investigation. The remaining 40 c.cm. were centrifuged and washed 3 times, the volume then made up to 40 c.cm. and 20 c.cm. withdrawn for subsequent study. The remaining 20 c.cm. were washed 5 times more, the volume made up to 20 c.cm. and 10 c.cm. withdrawn. Finally, the remaining 10 c.cm. were washed with 10 more changes of saline, and the precipitate, which had received 20 washings in all, was emulsified in 10 c.cm. of saline.

Mixture 11.—200 c.cm. of toxin + 3·25 c.cm. of antitoxin. 20 c.cm. of the mixture were withdrawn, and after it had stood for 1 hour, guinea-pigs were injected with 0·5 c.cm., 0·25 c.cm. and 0·1 c.cm. doses. This mixture was faintly opalescent after standing at room temperature overnight. The opalescence became more marked, and on the fourth day a very fine precipitate appeared, which settled very slowly to the bottom of the cylinder. The mixture was allowed to stand for 23 days, after which the supernatant fluid, which was

still faintly opalescent, was withdrawn, and the precipitate was washed 5 times and finally emulsified in 18 c.cm. of normal saline.

(*d*) For purposes of comparison a series of animals was injected with an ordinary toxin-antitoxin mixture, as prepared for use in general practice, and the antitoxin produced was measured in the same way as in all other experiments recorded in this paper.

The total nitrogen of these mixtures, and of the supernatant fluid and washed precipitates prepared from them in the manner described above, was determined, and thus a comparison could be made between the amount of nitrogen compounds injected and the antitoxin produced.

2. *General Plan of the Experiments.*

The antigenic activity of the whole mixtures, the supernatant fluid and the washed precipitates was investigated by injecting the various preparations into guinea-pigs and measuring the amount of antitoxin produced. Six guinea-pigs were used for the study of each preparation. All the animals used were bred in the Institute, and were kept under the same conditions and received the same diet throughout the experiment. Animals weighing from 250 gm. to 350 gm. were used.

Glenny and Südmersen (1921) have made a very complete study of the antitoxin production in guinea-pigs following a single injection of immunizing mixtures of varying composition. Among other findings they have shown that comparatively little antitoxin is produced during the first four weeks after injection, and that the maximum is reached eight weeks or more after injection; further, this maximum value may be retained a year or even longer. These workers have also shown that the amount of antitoxin produced by a single injection of toxin-antitoxin mixture varies, but is never very large (*i. e.* when compared with the amount produced in immunized animals after a secondary stimulus). Provided the mixture does not contain too large an excess of antitoxin, injected guinea-pigs respond by the production of amounts of antitoxin varying from 0·05 units to 0·5 units per c.cm. of serum. On rare occasions somewhat higher values were recorded.

As will be seen from the tables given below, these findings of Glenny and Südmersen with reference to the antitoxin production in guinea-pigs following a single primary stimulus have been confirmed.

About six weeks after the injection samples of blood were withdrawn from the inoculated animals and, after separation of the serum, antitoxin determinations were carried out. A second sample of blood was taken eleven to twelve weeks after inoculation and a second determination of antitoxin made. The values given are in terms of units of antitoxin per c.cm. of serum.

Antitoxin determinations were carried out by the intracutaneous method of Römer and Sames (1909). The samples of serum were first examined for the presence of a hundredth and a tenth of a unit per c.cm. of serum. Samples which contained less than a hundredth of a unit were tested for a two-hundredth of a unit, and those which contained less than this amount were tested for a five-hundredth of a unit. Tests for smaller quantities were not carried out. The samples of serum which contained more than a hundredth and less than a tenth of a unit were tested for the presence of a fiftieth and a twenty-fifth of a unit, and those which contained more than a tenth of a unit were tested for half a unit, and then for greater (0·6, 0·7, etc., units) or smaller (0·2, 0·3, 0·4 units) amounts. The antitoxin values obtained for the serum samples collected after 6 weeks gave a useful indication for the first bracketing tests made on the sample taken after 11 to 12 weeks. The figures entered in the tables below are minimal, and not necessarily exact, values; they indicate that the serum

contains at least the amount stated (*e.g.* 0·01 or 0·2 unit), and that less than the next higher value tested for (*e.g.* 0·02 or 0·3 unit) is present. In the case of nine samples the sign "<" ">" is prefixed, indicating that in these cases the stock of serum was exhausted before the tests, carried out in accordance with the scheme outlined above, could be completed.

RESULTS.

1. *The Toxicity for Guinea-pigs of the Mixtures 8, 9 and 11.*

With the exception of the last two entries in Table I below, the injections were made one hour after the toxin and antitoxin had been mixed together, and before flocculation had begun. The last two series of animals in the table, receiving 0·05 c.cm. and 0·02 c.cm. of Mixture 11, were injected three days after the mixture was made.

Assuming that the values found for the toxin and antitoxin used in this experiment are correct, then Mixture 8 should be innocuous to guinea-pigs in large doses since it is over-neutralized with antitoxin. This was actually found to be the case.

On the same assumption Mixture 9 should be exactly neutral. The tests on this mixture showed that the injection of 1 L_o dose of toxin mixed with 1 unit of antitoxin produced no effect on the animal: a volume of mixture containing ten times these quantities was tolerated and produced no detectable effect in six inoculated animals. A mixture containing twenty times the presumed L_o mixture caused late paralysis and death in nineteen to twenty-four days (six guinea-pigs). There is, therefore, a very slight excess of toxin in Mixture 9, which is revealed when large multiple doses of the (presumed) L_o mixture are injected.

Mixture 11 is a definitely toxic one. 0·1 c.cm. of the mixture contains an excess of toxin sufficient in amount to cause the death of guinea-pigs on the fourth day.

The results of these tests are collected in Table I:

TABLE I.

No. of mixture.	Dose injected. Volume (c.cm.).	Dose injected. Nitrogen (mgm.).	Number of guinea-pigs injected.	Results.
8	2·0	8·24	6	All lived; no local reaction.
9	2·0	7·84	6	All developed late paralysis; all died in 19 to 24 days.
	1·0	3·92	6	All lived; no reaction.
	0·1	0·392	2	Both lived; no reaction.
11	0·5	1·925	2	Died in 2 and 2½ days.
	0·25	0·962	2	Died in 2½ days.
	0·10	0·385	2	Died in 3½ and 4 days.
	0·05	0·192	1	Lived; medium swelling.
	0·02	0·077	6	Lived; slight local reaction.

2. *The Toxicity of the Washed Precipitates from the Toxin-Antitoxin Mixtures 8, 9 and 11.*

Guinea-pigs were injected with varying doses of the three preparations studied and the results are shown in Table II:

TABLE II.

No. of series.	Nature of preparation.	Dose injected. Volume (c.cm.).	Dose injected. Nitrogen (mgm.).	Number of guinea-pigs injected.	Results.
8 P.	Precipitate from mixture 8, washed 5 times	1·0	0·140	1	Lived; no reaction.
		0·2	0·028	6	Lived; no reaction.
9 P.	Precipitate from mixture 9, washed 5 times	1·0	0·115	1	Lived; slight thickening.
		0·2	0·023	6	Lived; no reaction
11 P.	Precipitate from mixture 11, washed 5 times	1·0	0·078	1	Died in 3½ days; P.M. typical; also inguinal glands enlarged and congested.
		0·2	0·0156	1	Marked local reaction; died on 9th day.
		0·1	0·0078	6	Definite local reaction; all animals developed late paralysis and died in 25 to 29 days.
		0·01	0·00078	6	Slight local thickening; no paralysis; all lived.
		0·001	0·000078	6	All lived; no local reaction.

In the case of the precipitates from, Mixtures 8 and 9, large doses, containing an amount of precipitate equivalent to that which separates from 10 c.cm. of original mixture, proved to be innocuous to guinea-pigs.

The washed precipitate from Mixture 11, however, is toxic for guinea-pigs when injected in large doses. 1 c.cm. of this preparation, which represents the precipitate from 10 c.cm. of original Mixture 11, caused the death of a guinea-pig in 3½ days; 0·2 c.cm. caused death in 9 days, and doses of 0·1 c.cm. caused late paralysis and death in 25 to 29 days. Smaller doses caused small local reactions (very small doses caused no local reaction), and the animals remained well and survived, and, as shown in Table III, developed a high degree of immunity.

3. *The Antitoxin Production Following Injection of Whole Mixture, Washed Precipitate and Supernatant Fluid.*

In Tables III, IV, V and VI below the antitoxin produced by the injection of toxin-antitoxin mixtures, washed precipitates from these mixtures, etc., are shown. The tables show the nature of the substance injected, its amount and nitrogen content, the interval between injection and the taking of the samples of blood for the antitoxin determinations, and the amount of antitoxin produced in units per cubic centimetre of serum.

In order to facilitate comparison of the antigenic value of the different preparations studied, it is convenient to arrange in one Table (III) the results showing the antitoxin produced by the injection of—

(1) Different mixtures of the same toxin and antitoxin.
(2) The washed precipitates from these mixtures.
(3) The filtered supernatant fluid remaining after the removal of the precipitate.
(4) A stock toxin-antitoxin mixture, prepared for general use.

TABLE III.

No. of series.	Nature of preparation.	Date of injection.	Dose injected. Volume (c.cm.).	Dose injected. Nitrogen (mgm.).	Number of guinea-pigs injected.	Interval between injection and bleeding, days.	Antitoxin produced (units per c.cm. of serum).
8	Toxin-antitoxin mixture	6 . iv . 25	2·0	8·24	6	40	0·005, <0·002, 0·005, 0·02, 0·02, 0·02.
						82	0·005, <0·002, <0·002, 0·04, 0·04, 0·02.
8 P.	Precipitate from mixture 8, washed 5 times	15 . iv . 25	0·2	0·028	6	41	0·005, 0·002, <0·002, 0·002, 0·01, 0·005.
						82	0·01, <0·002, 0·01, 0·01, 0·01, 0·002.
9	Toxin-antitoxin mixture	6 . iv . 25	1·0	3·92	6	42	0·04, 0·02, <0·01, <0·01, <0·01, 0·04.
						81	0·1, 0·02, 0·04, 0·04, 0·04, 0·2.
9 P.	Precipitate from mixture 9, washed 5 times	15 . iv . 25	0·2	0·023	6	41	0·02, >0·1, 0·01, 0·01, >0 1, 0·04.
						82	0·2, 0·6, died, 0·2, 0·5, 0·1.
9 S.	Supernatant fluid from mixture 9, filtered	15 . iv . 25	2·0	—	6	42	0·01, 0·005, 0·002, 0·02, 0·01, died.
						85	0·04, 0·01, 0·01, 0·04, 0·04, —.
11	Toxin-antitoxin mixture	15 . iv . 25	0·02	0·077	6	42	0·04, 0·2, 0·1, >0·2, 0·1, 0·04.
						78	0·5, 0·2, 0·1, 0·2, 0·2, 0·2.
11 G.	Precipitate from mixture 11, washed 5 times	7 . v . 25	0·01	0·00078	6	42	0·5, 0·2, 0·2, 0·2, 0·1, 0·04.
						79	0·3, 0·2, 0·5, 0·2, 1·3, 0·2.
1018	Stock toxin-antitoxin mixture	15 . iv . 25	2·0	1·078	6	41	0·005, 0·005, 0·005, 0·01, 0·005, 0·002.
						85	0·02, 0·005, 0·01, 0·04, 0·01, 0·005.

The experiments described in this paper are concerned in the main with the antigenic value of toxin-antitoxin precipitates. It appeared desirable, however, to investigate the antigenic properties of the mixtures which furnished these precipitates, and in Table III above the antigenic properties of three such mixtures are shown and can be compared with those of the three washed precipitates which separated from these mixtures.

As regards the antigenic properties of the original toxin-antitoxin mixtures, the results illustrate the well-known fact that the antigenic value of such mixtures is closely related to the degree to which the toxin is neutralized. Park has issued repeated warnings against over-neutralization, since the immunizing value of such mixtures is relatively small. In Table III the six animals which received 2 c.cm. doses of Mixture 8, which contains antitoxin in excess, developed relatively little antitoxin; the six animals which received 1 c.cm. doses of Mixture 9, which is an almost exactly neutral mixture, produced more antitoxin, while the 6 animals which received only 0·02 c.cm. quantities of Mixture 11, which is markedly toxic, produced relatively large quantities of antitoxin.

The figures given in Table III also demonstrate that the antigenic value of a washed toxin-antitoxin precipitate varies with the nature of the mixture

from which the precipitate separates. Mixture 8 was over-neutralized with antitoxin, and the precipitate from this mixture is a relatively poor antigen. The precipitate from the neutral Mixture 9 is a much better antigen, and the precipitate from the toxic Mixture 11 is a remarkably active antigen. Six guinea-pigs injected with a very small dose of this preparation, containing in fact less than a thousandth of a milligramme of nitrogen, responded by the production of amounts of antitoxin amounting to as much as 0·2 to 1·3 units per cubic centimetre of serum. Other results illustrating the antigenic activity of this particular preparation are given below. It may be pointed out here that these results confirm those recorded in an earlier paper in which different toxins and antitoxins were used, and further, the suggestion made then that the precipitates prepared from toxic mixtures of toxin and antitoxin would exhibit antigenic activity of a high order is borne out by the experiments described above, and elsewhere in this paper.

A comparison can also be made between the antigenic value of the whole toxin-antitoxin mixture and the washed precipitate which separates from such a mixture, and this comparison is of particular interest and significance when the nitrogen content of the whole mixtures and the precipitates which separate from the mixtures are also compared. 2·0 c.cm. of the over-neutralized Mixture 8, containing 8·24 mgm. of nitrogen, gave approximately the same immunity response as the precipitate which separated from this volume of mixture; the nitrogen content of the precipitate, however, is only 0·028 mgm., or one three-hundredth of the amount of nitrogen contained in the whole mixture. In the case of the precipitate from the toxic Mixture 11, the injection of an amount of precipitate equivalent to that which separates from 0·1 c.cm. of original mixture, and containing only 0·00078 mgm. of nitrogen, resulted in the production of antitoxin in amounts comparable with those produced by the injection of 0·02 c.cm. of original mixture, containing 0·077 mgm. of nitrogen—or 100 times as much as the precipitate.

The results obtained with the neutral Mixture 9, the precipitate from this mixture, and the fluid remaining after the precipitate had been separated, are of special interest, and suggest that the large amount of foreign nitrogenous compounds normally occurring in toxin-antitoxin mixtures actually interfere with the immunizing response to the specific antigen. In these experiments six animals which received 1 c.cm. of the whole Mixture 9, containing 3·92 mgm. of nitrogen, developed amounts of antitoxin varying from 0·02 to 0·2 units per c.cm.; five animals which received an amount of precipitate equivalent to that which separates from 2 c.cm. of this mixture, and containing only 0·023 mgm. of nitrogen, developed from 0·1 to 0·6 units of antitoxin per c.cm.; while on the other hand five animals which received 2 c.cm. doses of the supernatant fluid remaining after the precipitate had been removed, and containing nearly 8 mgm. of nitrogen, developed relatively little antitoxin, viz. from 0·01 to 0·04 units per c.cm.

The figures showing the antitoxin produced by the injection of 2 c.cm. of the stock toxin-antitoxin mixture into 6 guinea-pigs are also included in Table III for purposes of comparison. From the amount of nitrogen which this preparation contains it would appear to belong to the later type of mixture, containing 0·1 L_+ dose of toxin, the use of which is recommended by Park,

Schroder and Zingher (1923). As will be seen, the antitoxin production following the injection of this preparation is relatively small. A strict comparison with any of the whole mixtures (8, 9 and 11) used in these experiments cannot easily be made, since the amounts of nitrogen which these antigens contain vary rather widely. The figures for the antitoxin produced by injection of 2 c.cm. dose of the stock mixture resemble most closely, perhaps, those shown for the supernatant and filtered fluid from Mixture 9, although it must be pointed out that this fluid contains some seven times as much nitrogen as the stock mixture.

4. *The Effect of Repeated Washing on the Nitrogen Content and Antigenic Activity of a Toxin-Antitoxin Precipitate.*

The method by which the reagents for this experiment were prepared is described above (p. 56). The precipitate from Mixture 9 was used, and the effect of washing with 2, 5, 10 and 20 changes of normal saline was investigated. The results are collected in Table IV:

TABLE IV.

No. of series.	Nature of preparation.	Date of injection.	Dose injected. Volume (c.cm.).	Dose injected. Nitrogen (mgm.).	Number of guinea-pigs injected.	Interval between injection and bleeding, days.	Antitoxin produced (units per c.cm. of serum).
9 B.	Precipitate from mixture 9, washed twice	24 . iv . 25	0·2	0·024	6	84	0·2, 0·4, 0·1, 0·3, 0·7, 0·2.
9 P.	Precipitate from mixture 9, washed 5 times	15 . iv . 25	0·2	0·023	6	82	0·2, 0·6, died, 0·2, 0·5, 0·1.
9 D.	Precipitate from mixture 9, washed 10 times	24 . iv . 25	0·2	0 021	6	84	0·1, 0·5, 0·7, 0·04, 0·1, 1·2.
9 E.	Precipitate from mixture 9, washed 20 times	24 . iv . 25	0·2	0·019	6	84	0·2, 0·2, 1·2, 0·1, 0·3, 0·1.

A slight decline in the nitrogen content of the preparation accompanies the process of washing. This may be due to solution of nitrogen compounds or to mechanical loss during the operations. The antitoxin production is substantially the same throughout the series, and it is evident that whatever the nature of the antigen may be, and whatever the manner of its association with other ingredients of the precipitate, the combination is a firm and stable one and is not dissociated by washing with normal saline.

The result is in agreement with the observations of Ramon (1923), who remarks upon the insolubility of toxin-antitoxin precipitates in salt solution. Ramon states, however, that the toxin and antitoxin can be dissociated and the precipitate brought into solution by means of very dilute acetic acid, and that the careful heating of such an acid dissociated solution destroys the toxin and

leaves a concentrated, highly purified solution of antitoxin. 75 per cent. of the antitoxin units originally present in the mixture may be recovered in this way.

5. *The Effect of the Amount of Antigen Injected on the Antitoxin Produced.*

The effect of varying the amount of toxin-antitoxin precipitate injected on the antitoxin produced has been investigated, two different preparations being used.

Earlier experiments on this point had shown that the immunity response was apparently the same to small as to large doses of these washed precipitates. Glenny and his colleagues (1926) also found that the injection of a similar preparation, made and studied by them, in doses varying from 1·5 c.cm. to 0·01 c.cm., produced the same degree of immunity, and that doses of 0·001 c.cm. were scarcely less efficient. These results may be explained, perhaps, on the assumption that the minimum immunizing dose of these washed precipitates is very small, and in the experiments carried out hitherto this limiting value, or dose, had not been reached.

The results of these Experiments are given in Table V:

TABLE V.

No. of series.	Nature of preparation.	Date of injection.	Dose injected. Volume (c.cm.).	Dose injected. Nitrogen (mgm.).	Number of guinea-pigs injected.	Interval between injection and bleeding, days.	Antitoxin produced (units per c.cm. of serum).
9G.	Precipitate from mixture 9, washed 5 times	24.iv.25	1·0	0·115	6	41 83	0·04, 0·01, 0·02, 0·04, 0·04, 0·04. 0·7, 0·02, 0·1, 0·1, 0·2, 0·3.
9P.	Ditto	15.iv.25	0·2	0·023	6	41 82	0·02, >0·1, 0·01, 0·01, >0·1, 0·04. 0·2, 0·6, died, 0·2, 0·5, 0·1.
9H.	„	24.iv.25	0·02	0·0023	6	42 83	0·01, 0·02, 0·01, 0·01, 0·01, 0·01. 0·1, 0·04, 0·01, 0·01, 0·01, 0·01.
9K.	„	24.iv.25	0·002	0·00023	6	42 83	0·01, 0·002, 0·04, 0·01, 0·01, 0·01. 0·04, 0·04, 0·04, 0·02, 0·04, 0·1.
11G.	Precipitate from mixture 11, washed 5 times	7.v.25	0·01	0·00078	6	42 79	0·5, 0·2, 0·2, 0·2, 0·1, 0·04. 0·3, 0·2, 0·5, 0·2, 1·3, 0·2.
11H.	Ditto	7.v.25	0·001	0·000078	6	42 79	0·01, 0·02, 0·01, 0·02, 0·1, 0·04. 0·02, 0·04, 0·04, 0·04, 0·04, 0·1.

In both cases a relationship between the size of the dose and the antitoxin produced is observed. In the case of the precipitate from Mixture 9, the antitoxin production following the injection of 1·0 c.cm. and 0·2 c.cm. doses of the preparation is of the same order, and it will be observed that of the eleven surviving animals which were injected with these amounts only one failed to produce as much as 0·1 unit of antitoxin, while three others produced as much as 0·5, 0·6 and 0·7 units respectively. The antitoxin production

following the injection of much smaller doses of the same preparation, viz. 0·02 c.cm. and 0·002 c.cm., is much less; of the twelve animals receiving these amounts only two produced as much as 0·1 unit, while the remaining ten produced from 0·01 to 0·04 units. The difference between these two groups is quite definite, but the relationship between dosage and antitoxin production is not absolutely regular; the six animals which received the smallest dose produced on the whole slightly more antitoxin than the group receiving a dose ten times as large.

In the case of the washed precipitate from Mixture 11 the influence of the size of the dose on the antitoxin produced is well shown. Six animals which received 0·01 c.cm. of the preparation produced from 0·2 to 1·3 units of antitoxin, while of the six which received 0·001 c.cm. of antigen only one produced as much as 0·1 unit, and the remaining five amounts varying from 0·02 to 0·04 units.

6. *The Effect of the Route of Administration of Washed Toxin-Antitoxin Precipitates on Antitoxin Production.*

Sordelli and Serpa (1925) have studied the antigenic activity of unwashed precipitates from toxin-antitoxin mixtures. They immunized rabbits and guinea-pigs by intracutaneous injection and also by scarification. They state that a solid active immunity followed two scarifications and applications of precipitate, and consider that the material and the scarification method can be employed for the active immunization of man.

A series of experiments similar to those described by Sordelli and Serpa have been carried out, using two different washed toxin-antitoxin precipitates. Groups of six guinea-pigs were injected with the same preparation, the animals of one group being injected intracutaneously, and those of the other group receiving the same dose of the antigen applied to scarified areas on the shaven flank of the guinea-pigs.

It is practically impossible to administer an accurately measured dose of antigen by the scarification method, and the results obtained accordingly lose a good deal of their quantitative significance. The operation was carried out in the following way: White or cream-coloured guinea-pigs were shaven and anæsthetized. A number of small areas, about 0·5 cm.^{2}, were scratched with a sharp scalpel held obliquely. The attempt was made to avoid penetrating the deeper layers of the skin and to cause bleeding, but these attempts were not always successful. Small drops of the antigen preparation were then placed on the scarified areas by means of a 0·1 c.cm. capillary pipette and the drop spread out over the scratched area with the end of the pipette. The anæsthesia was maintained until the material was quite dry.

Antitoxin determinations were carried out in the usual way and the results are given in Table VI.

The effect produced on the animal by the intracutaneous injection of these two preparations was markedly different. The precipitate from the neutral mixture, 9, on intracutaneous injection, caused a very slight local reaction which faded rather quickly; there was no scaling of the skin and the hair-growth was not retarded. No detectable reaction followed the application of

this preparation to the scratched areas of the skin. The intracutaneous injection of the washed precipitate from the toxic mixture 11, however, was followed by a large and severe local reaction, followed by necrosis of the skin. The large scabs which formed ultimately sloughed off and the lesion healed, but the hair did not begin to grow again for several weeks. In other respects the animals remained well; they increased in weight and none of them showed signs of paralysis. The scarified areas to which the precipitate from Mixture 11 was applied showed small local reactions of varying intensity, some being well marked the day following the operation, but all were very much faded and reduced on the third day after application.

TABLE VI.

No. of series.	Nature of preparation.	Date and route of injection.	Dose injected. Volume (c.cm.).	Dose injected. Nitrogen (mgm.).	Number of guinea-pigs injected.	Interval between injection and bleeding, days.	Antitoxin produced (units per c.cm. of serum).
9 L.	Precipitate from mixture 9, washed 10 times	7.v.25 Intracutaneous	0·2	0·021	6	43	0·02, <0·002, 0·02, 0·02, 0·005, 0·04.
						79	0·2, 0·01, 0·1, 0·1, 0·005, 0·04.
9 M.	Ditto	7.v.25 Scarification	0·2	0·021	6	43	<0·002, <0·002, <0·002, 0·002, 0·01, 0·02.
						79	0·005, 0·002, <0·002, 0·005, 0·02, 0·01.
11 L.	Precipitate from mixture 11, washed 5 times	7.v.25 Intracutaneous	0·1	0·0078	6	43	0·2, 0·2, 0·2, >0·5, >0·5, 0·2.
						80	0·4, 0·5, 0·5, >2·0, 0·5, 0·7.
11 M.	Ditto	7.v.25 Scarification	0·1	0·0078	6	43	0·04, 0·01, 0·04, 0·04, 0·04, 0·02.
						80	0·5, 0·1, 0·1, 0·5, 0·1, 0·04.

The results of the intracutaneous injection and application to the scarified skin of these preparations are in accord with the properties of the washed precipitates, as recorded in Table II.

The antitoxin production following the injection, by different routes, of the two preparations shows an interesting contrast. The precipitate from the neutral mixture 9, when injected intracutaneously produced as much as 0·2 unit of antitoxin per c.cm. of serum in one case and 0·1 unit in two others. When applied to the scarified skin, however, very little antitoxin was produced in four animals, but in the remaining two 0·01 and 0·02 units per c.cm. were produced. On the other hand, the antitoxin produced by the intracutaneous injection of the precipitate from Mixture 11 was very large, the serum of one animal containing more than 2 units per c.cm. The antitoxin production following the application of this preparation to the scarified skin was also relatively large; two animals produced 0·5 unit and three others 0·1 unit per c.cm. of serum. How much of the effect produced in this group is due to absorption of the antigen at those places where the deeper layers of the skin were penetrated can only be conjectured.

Sordelli and Serpa state that the degree of immunity following intracutaneous injection is greater than that following the application of the antigen to the scarified skin, and the results of the above experiments confirm

those of Sordelli and Serpa. The results show, further, that the amount of antitoxin produced after intracutaneous or cutaneous injection varies with the nature of the antigen, the toxic preparation being much more effective than the one prepared from the neutral mixture.

The biological action of the washed precipitates resembles that of toxin-antitoxin mixtures in so far as injection into guinea-pigs is followed by the production of a state of active immunity and the appearance of antitoxin in the circulating blood. The two antigens differ materially, however, in their composition and physical properties. Toxin-antitoxin mixtures contain the active antigen in the dissolved condition, but mixed with large amounts of different soluble organic nitrogen compounds—the proteins of horse-serum, the products of protein hydrolysis, the nitrogenous extractives of muscle and non-specific, and possibly antigenic, substances of bacterial origin. The washed precipitates, on the other hand, contain the antigen in an insoluble form, and, moreover, in a relatively pure state, since over 99 per cent. of the extraneous foreign substances have been removed during the preparation of the antigen. The course of events following the injection of solutions of antigen, accompanied by the simultaneous injection of a large excess of soluble organic nitrogen compounds of very varying composition, may be somewhat different from the course of events following the injection of the same antigen in an insoluble form and unaccompanied by such a large excess of impurities. It is also conceivable that the reaction between the cells and a soluble antigen, such as is represented by a toxin-antitoxin mixture, takes place more quickly than in the case of the washed precipitate from such a mixture, since the antigen occurring in the latter must, presumably, first be brought into solution, either by the action of the leucocytes or the body-fluids. Another factor, which Glenny believes to be of importance in determining the course and extent of immunization in diphtheria, is that of dissociation. He considers that the efficiency of toxin-antitoxin mixtures is due to the slow dissociation of the toxin and antitoxin, so that the animal is constantly stimulated. Glenny and his co-workers have produced a good deal of experimental evidence in support of this view.

It is suggested that the antigenic efficiency of the washed precipitates is due in part to the relative purity of the preparation, and in part to the relatively slow solution and absorption and slow dissociation in inoculated animals, which results in a long-continued stimulus being applied and the production of a high degree of immunity.

Schmidt and Scholz (1925) have recently carried out investigations on somewhat similar lines to those described above. In their experiments rabbits were used. They found that three rabbits which received repeated injections of increasing doses of antigen developed from 0·02 to 0·2 units of antitoxin per c.cm. of serum, while four other rabbits, which received one injection only, developed from 0·02 to 0·2 units of antitoxin per c.cm. of serum eleven weeks after injection. From their experiments and from results obtained on man in Marburg by Prof. Schreiber, Schmidt and Scholz consider that these washed toxin-antitoxin precipitates constitute the best antigen as yet available for the active immunization of man. They state that the injection is painless, that the local reaction following the injection of 0·3 to 0·4 c.cm. of their preparation

is trifling or absent altogether, and that at least 0·1 unit of antitoxin per c.cm. of serum is produced in inoculated subjects.

SUMMARY AND CONCLUSIONS.

(1) Using the same diphtheria toxin and antitoxin, an over-neutralized, a neutral and an under-neutralized (toxic) mixture have been prepared. The antigenic properties of the washed precipitates which separated from these mixtures have been investigated.

(2) Mixtures containing an excess of toxin flocculate slowly, and the amount of precipitate which ultimately separates is less than in the case of neutral or over-neutralized mixtures of the same reagents.

(3) The toxic and antigenic properties of these precipitates bear a close relationship to the nature of the mixture from which they are derived. The precipitate from the over-neutralized mixture was atoxic and its antigenic activity was small; the precipitate from the neutral mixture was also devoid of toxicity, but it was a good antigen; the precipitate from the toxic mixture was itself toxic when injected in relatively large doses, and it was also a remarkably active antigen.

(4) The precipitates contain less than 1 per cent. of the total nitrogen present in the mixtures from which they separate. The most active preparation studied in this series contained only one five-hundredth (0·02 per cent.) of the total nitrogen occurring in the mixture from which it was derived.

(5) In the case of the neutral mixture it has been shown that most of the antigenic activity is associated with the precipitate; the fluid remaining after removal of the precipitate possesses relatively little antigenic activity. Some evidence has been obtained which suggests that the large amount of extraneous nitrogenous compounds which toxin-antitoxin mixtures contain interferes with the production of antitoxin.

(6) Repeated washing of toxin-antitoxin precipitates with normal saline produces but little change in the nitrogen content of the preparation, and appears to be without effect on its antigenic activity.

(7) Antitoxin production follows the injection of very small amounts of those precipitates which separate from neutral or toxic mixtures. Thus, injections of amounts of precipitate prepared from a neutral mixture, containing a four-thousandth of a milligramme of nitrogen, produced from 0·02 to 0·1 units of antitoxin per c.cm. of serum in six guinea-pigs. Doses a hundred times as large produced from 0·1 to 0·6 units. The precipitate from a toxic mixture was even more active: doses containing less than a ten-thousandth of a milligramme of nitrogen produced from 0·02 to 0·1 units of antitoxin per c.cm. of serum in inoculated animals, and doses ten times as large produced from 0·2 to 1·3 units.

(8) Antitoxin production follows the intracutaneous injection of washed precipitates; it has also been shown that application of the washed precipitate to the scarified skin of guinea-pigs is followed by the production of antitoxin in the blood, although in smaller amounts. As in the case of subcutaneous injection, the amount of antitoxin produced varies with the nature of the antigen.

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VOLUMETRIC MEASUREMENT BY DROPS IN BACTERIOLOGICAL TECHNIQUE.

P. FILDES* AND W. A. M. SMART.

From the Bacteriological and Physiological Departments, London Hospital.

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DONALD (1913, 1915, 1916) introduced into bacteriology a method of measuring small volumes of liquids by means of drops. It depended upon the fact that the size of a drop delivered from a pipette is, other factors being constant, governed by the *external* diameter of the dropping tube.

The method is usually applied by fitting the pipette into a standard steel wire gauge and cutting the pipette at the point of impaction. Any pipette gauged in the same hole will be of equal external diameter, and will then deliver equal volumes of water if held in the same (vertical) position. The dropping is supposed to be carried out in a room at the same, or nearly the same, temperature, and the rate of drops must be slower than 60 per minute. The volumes

* Working on behalf of the Medical Research Council.

of drops of other liquids than water are arrived at by applying a coefficient represented by the ratio—

$$\frac{\text{number of drops of fluid per c.cm.}}{\text{number of drops of water per c.cm.}}$$

The present investigation is an attempt to improve on the accuracy of Donald's technique for comparison with methods of measurement by volumes (Fleming, 1924).

THE RELATION OF DROP-RATE TO DROP-WEIGHT.

Donald (1913) counted the number of drops of water required to make a certain volume at different rates. It was found that a larger number was required as the rate decreased, and he concluded that " these small drops have

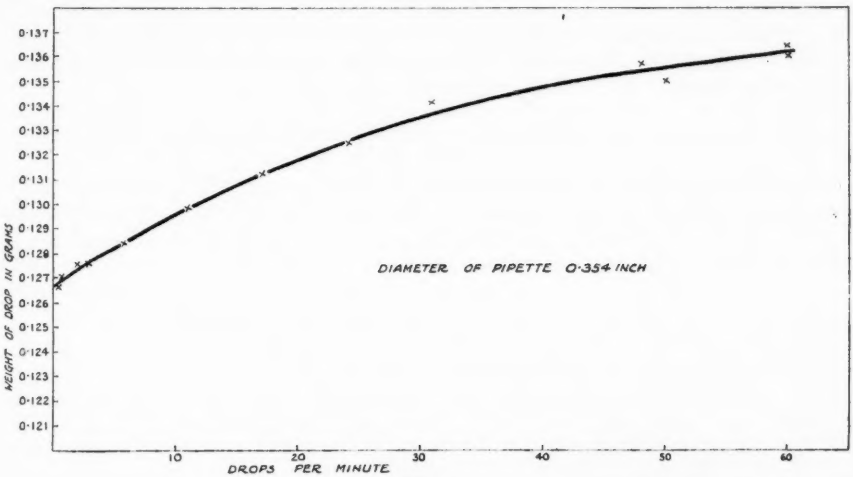

FIG. 1.

in their favour the peculiarity (pointed out by Ollivier) of being practically constant in weight at all expulsion rates slower than one drop per second." This point was investigated by us as follows:

Ten brass pipettes were made with flat ends and vertical sides. Their diameters ranged from 23 mm. to 1 mm., the error in their diameters being within $\frac{1}{100}$ mm. They were mounted on the constant-head dropping apparatus used by Donald, an adaptation of a Mariotte's tube. In order to obtain constant results, it is necessary to make certain that the drop is properly formed, and hence of perfect shape. For this purpose, the lower face of the pipette must be horizontal, so that the drop is suspended from the extreme circumference of the tube. To make sure that the whole contour of the face was covered, the pipette was heated in the flame and cooled before use. Such fluctuations as occurred in the temperature of the room appeared to have no demonstrable effect.

A number of observations were made with each pipette, consisting in the determination of the weight of ten drops of water at different drop-rates. Sensitivity of the balance; 0·001 gm. = 1 division on the scale. The calculated weight of one drop was then plotted against the rate of fall (drops per minute). Fig. 1 shows a typical curve (out of ten) obtained by the above method. It is approximately a parabola, the weight of a drop slowly increasing with increase in drop-rate. At the slower rates a curve could be well fitted to the observations, but at faster rates the readings became increasingly erratic and the "fit" of the curve was somewhat problematical. The variations were found to be due to an increasing distortion of the shape of the drop. With small pipettes the weights tend to become irregular at about 60 per minute, and with the larger ones at rates even as slow as 30 per minute.

The curves were then studied from the point of view of Donald's conclusion. It was found that whatever the size of the tube, the weight of the drop, at 50 per minute, was about 4 per cent. larger than at 10 per minute. The statement, therefore, that at rates under 60 per minute the weight is almost constant cannot be taken to hold when accuracy is required.

THE RELATION OF THE DROP-WEIGHT TO THE EXTERNAL DIAMETER OF THE TUBE.

It is evident that a curve showing this relationship may be obtained for any given specified rate of dropping. This has been done by Lord Rayleigh (1899), who worked with ten drops following one another at intervals of about fifty seconds. His results did not, however, include many of the lower-sized

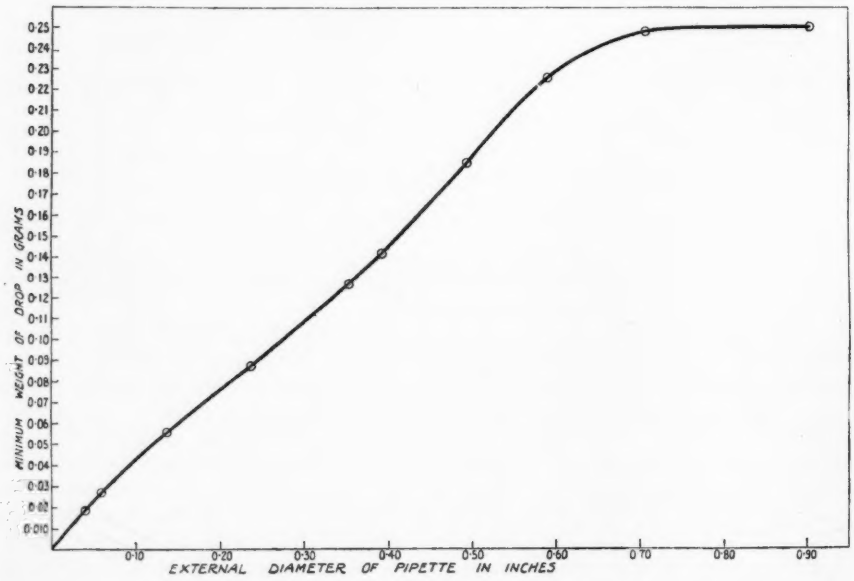

FIG. 2.

tubes such as are of frequent use in bacteriological technique, and the graph was not completely drawn. By extrapolation from each of our weight-rate curves we can determine the minimum-sized drop, *i.e.* one which is formed infinitely slowly. By plotting the smallest possible drop for each tube, the errors of observation are greatly reduced. This relation is shown in Fig. 2. On examination it shows that there is a steady and continuous rise in the minimum weight, as the external diameter of the tube increases. Ultimately this rise begins to fall off, leading to a more or less flat top, or more correctly it ends up asymptotically. This demonstrates that the maximal "minimum drop" is attained by a tube of diameter just over 0·9 inches. Each of the ten brass pipettes accounts for one of the points plotted on the curve.

More minute examination of the curve shows that the rise is not uniform, and has two points of inflection. The curve is first concave to the x axis, then becomes convex, once more gets concave, and finishes by running almost parallel to it. This indicates some alterations in the statico-dynamics of the formation of the drop, about which very little appears to be known. It may, however, be noted that in the lower concave part of the curve the diameter of the drop is greater than that of the pipette, and that during the formation of the drop the water creeps up the sides of the pipette. On the other hand, in the convex and upper concave parts the diameter of the drop is smaller than that of the pipette and no creeping takes place. Where the lower concave part of the curve passes into the convex part the diameters of drop and pipette are equal.

CORRELATION OF DROP-WEIGHT, DROP-RATE AND SIZE OF PIPETTE.

The information derived from the drop-rate and minimum drop-weight curves was now amalgamated to construct a "nomogram" (Smart, 1923)—that is, a diagram which seeks to correlate all three measured quantities. From a theoretical point of view one should construct it from a knowledge of the formulæ of the curve already obtained. As, however, the dynamical basis is unknown we cannot do so, with the result that perforce the nomogram has had to be constructed empirically. None the less it is quite reliable in the information it gives. Fig. 3 is a nomogram showing the full relation between the above-mentioned quantities for all sizes of pipettes. The upper line is an equally divided scale showing the weights of water delivered, and the lower line, also equally divided, shows the rate of dropping (from none to 60 drops per minute). The intermediate curve is marked out to correspond with any size pipette lying between zero and 0·9 inches, and divided to show tenths. It was obtained by drawing a freehand curve through a set of ten points determined in the following way: The weight-rate curve for each one of the brass pipettes was taken, and the readings for any two rates were noted—say zero and 60 per minute. Each of these values was marked on the top line of the nomogram, and joined to the corresponding rate on the base line. The intersection of these lines gives a point through which all measurements for that particular pipette must pass. It is therefore a fixed point, and can be labelled with the diameter of the pipette to which it belongs. Proceeding in a similar way with the other tubes, one thus obtains a set of ten points from which the curve may be drawn with sufficient accuracy.

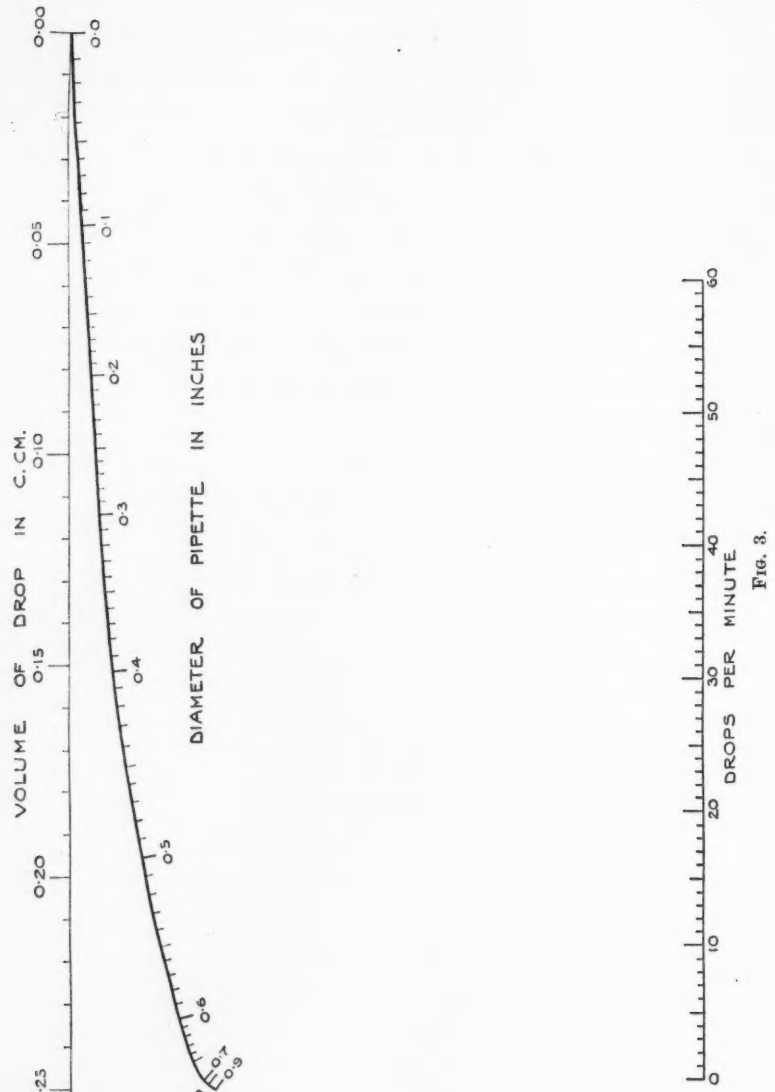
VOLUME OF DROP IN C.CM.
0·00
0·05
0·10
0·15
0·20
0·25
DIAMETER OF PIPETTE IN INCHES
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
DROPS PER MINUTE
0
10
20
30
40
50
60

FIG. 3.

The great merit of such a nomogram, as it is called, is that by joining up any two of the variables the line so drawn automatically cuts the third at the point corresponding to the required value of that variable. For example, if we join the weight of water required to be delivered (per drop) with any convenient size of pipette, the intersection of the line with the third quantity gives at once the rate of dropping necessary.

PRACTICAL APPLICATIONS.

The Measurement of Large Quantities.

The volumes delivered by pipettes of large size at different rates may be obtained from Fig. 3.

When first making the measurements already recorded we used glass pipettes, cut square, and ground smooth on a fine stone. The effective diameter was taken to be the mean of the largest and smallest as measured by a micrometer caliper. It was not, however, found possible to secure sufficient uniformity in the results, and the glass tubes were discarded for brass ones.

For most purposes the measurement of large quantities by means of single drops has no special advantage over other methods. The apparatus is somewhat cumbersome and not easily controlled. If it is used, it would appear necessary, for accurate work, to use a series of brass pipettes of known diameter. We have not observed any constant difference between the weights of drops delivered from glass or brass tubes.

The Measurement of Small Quantities.

The practical value of the drop method of measurement depends on the facility with which small known amounts may be delivered from a pipette held in the hand. This mode of manipulation is only applicable to tubes under a certain size. We have found that the variation in size of hand-delivered drops becomes much more marked with pipettes greater than 0·1 inches diameter. This, therefore, gives an upper limit to the size of tube which may be used with a hand-dropping method.

The general technique we have used follows closely upon that described by Donald.

Fig. 4 shows that part of Fig. 3 which is of practical use. It has been sufficiently enlarged to allow of the estimation of volumes to a fraction of a cubic millimetre. The pipette scale is calibrated in gauge plate numbers instead of inches, the diameters of the numbered holes being stated by the makers to $\frac{1}{1000}$ in. The largest pipette included is Morse 36, which corresponds to the largest size giving uniform results with hand-dropping. The smallest pipette, No. 60, is the smallest included in the ordinary gauge-plates. The scale marked " Stubs " refers to Stubs's twist drill and steel wire gauge plate, while that marked " Morse " is a similar one for Morse sizes.

The readings obtained from this nomogram will only be accurate under certain definite conditions:

1. *The accuracy of the gauge plates used.*—Although the diameter of the holes in gauge-plates is given to $\frac{1}{1000}$ in., and in some cases to $\frac{1}{2000}$, it was found on actual measurement with a microscope stage micrometer that there

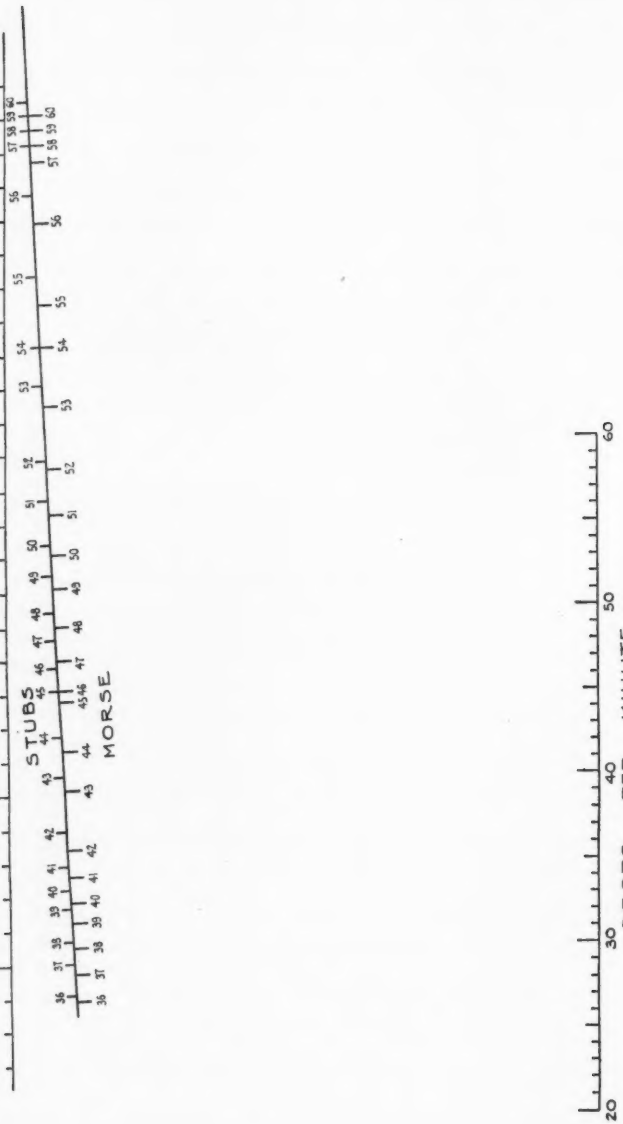
C. CM. DELIVERED
0·045
0·040
0·035
0·030
0·025
0·020
STUBS
MORSE
DROPS PER MINUTE
20
30
40
50
60

Fig. 4.

was a surprising degree of error. The stage micrometer itself showed no appreciable error, and in any case its error affected each of the three gauges measured. The results are shown in Table I:

TABLE I.—*Accuracy of Steel Wire Gauges.*

Twist drill and steel wire gauge, No. 705, Brown & Sharpe Mfg. Co., Providence, U.S.A. (Morse sizes). The measurements are in inches:

No. 57.	Stated size 0·043	Observed size 0·0429.
No. 54.	,, ,, 0·055	,, ,, 0·0550.
No. 51.	,, ,, 0·067	,, ,, 0·0666.

Stubs's twist drill and steel wire gauge:

No. 57.	Stated size 0·042	Observed size 0·0456.
No. 54.	,, ,, 0·055	,, ,, 0·0596.
No. 51.	,, ,, 0·066	,, ,, 0·0694.

Twist drill and steel wire gauge (Buck & Hickman):

No. 57.	Stated size 0·043	Observed size 0·0446.
No. 54.	,, ,, 0·055	,, ,, 0·0582.
No. 51.	,, ,, 0·067	,, ,, 0·0701.

It should be stated that the Buck & Hickman gauge was not new, and possibly the holes may have been expanded in use. The particular holes referred to in the Stubs gauge had not, however, been used before. The Brown & Sharpe gauge was new, and of a high degree of accuracy. The order of error in the Stubs gauge is sufficient to account for an error of 2 or 3 cubic millimetres in reading the nomogram. When, therefore, the nomogram is used, the correctness of the holes should be verified.

2. *The accuracy of the measurement of the pipette by the gauge plate.*—When testing the readings of the nomogram, it was found that the volume delivered by a glass pipette measured with a Brown & Sharpe gauge was always rather over 0·5 c.mm. smaller than that indicated by the nomogram. We concluded that this was due to the fact that the largest diameter of the pipette was measured by the gauge plate, while the effective diameter was smaller owing to the pipettes not being round. Since this error was roughly constant, we have corrected it in Fig. 4 by moving the volume scale 0·5 c.mm. to the left.

3. *The accuracy of measurement of the drop-rate.*—The uniformity of the drop-rate may be obtained by two methods:

(*a*) The micro-Mariotte tube: This apparatus, shown in Fig. 5, is made by drawing out a piece of 7 mm. glass tubing to make a pipette with rather square shoulders. The stem is cut away from the barrel, and the dropping points, about 1½ in. long, are attached by a piece of bicycle valve tubing. The capillary which is thrust through the tip of the teat should be wide enough to prevent any capillary action. The

FIG. 5.

upper end of the capillary is drawn out very finely, and is sealed with wax into another short barrel after the manner of a Wright's throttled pipette. The "head" of the apparatus should be kept constant by adjusting the orifice of the internal tube to a constant distance (2½ in.) from the orifice of the pipette. The pipette is dipped into the liquid to be measured, and while the upper barrel is kept securely closed with the finger, the teat is compressed and the lower barrel filled. On removing the finger air will now enter through the throttle, and allow drops to emerge at a constant rate. By carefully nipping off short lengths of the fine capillary, the rate of dropping with a medium-sized pipette may be adjusted to about 30 per minute. The micro-apparatus may be kept permanently with a note of its drop-rates with different points, but it is not easily cleaned, and can hardly be sterilized. It is essential to avoid compressing the teat without a finger on the upper orifice, otherwise the throttle will become choked. The drops are, of course, stopped or started by means of the finger on this orifice.

(*b*) Hand compression of a teat: With very little practice drops may be delivered at a sufficiently constant rate from an ordinary Pasteur pipette fitted with a teat and compressed with the fingers. By such a method, though the expulsion rate of the drop may be constant, the formation of the drop is not continuous. The impulses of the fingers are intermittent. Nevertheless Table II shows that the practical results of hand-dropping are very little more erratic than those of automatic dropping.

TABLE II.—*Volume of One Drop of Water (Calculated from that of Ten Drops) Delivered from the same Pipette at about 30 Drops Per Minute by the Two Methods.*

Micro-Mariotte.		Hand.	
0·0266 c.c. 0·0267 „ 0·0266 „ 0·0265 „ 0·0266 „	Maximum variation, 0·0002 c.c.	0·0267 c.c. 0·0266 „ 0·0267 „ 0·0269 „ 0·0264 „	Maximum variation, 0·0005 c.c.

For most purposes in bacteriological work the micro-Mariotte pipette would be impracticable. Therefore, to obtain accuracy the worker must train himself to deliver drops by hand from any pipette at a more or less constant rate. This should not be greater than 50 per minute (preferably about 30), owing to the erratic behaviour of drops at fast rates already referred to.

For comparison with the drops delivered at 30 per minute, the following readings are shown with the same pipette operated by hand at 50: 0·0274 c.c., 0·0274 c.c., 0·0273 c.c., 0·0271 c.c., 0·0275 c.c. Drops delivered at 50 are therefore about 2½ per cent. larger than those at 30.

In order to test the general accuracy of the technique and of the nomogram, eight different pipettes were then made and measured with a Brown & Sharpe gauge. The volume of 1 drop from each was estimated from the weight of 10 delivered from an automatic pipette (Table III).

TABLE III.—*Observed Error with Pipettes of Different Diameter.*

Number.	Rate.	Observed.	Calculated.	Error.
54	33	0·0255 c.c.	0·0257 c.c.	−0·0002 c.c.
52	32	0·0292 „	0·0292 „	0·0 „
50	32	0·0319 „	0·0319 „	0·0 „
47	30	0·0350 „	0·0352 „	−0·0002 „
44	29	0·0381 „	0·0380 „	+0·0001 „
42	28	0·0405 „	0·0411 „	−0·0006 „
39	25	0·0430 „	0·0433 „	−0·0003 „
36	24	0·0454 „	0·0458 „	−0·0004 „

The maximum error in these observations is therefore about 1½ per cent.

4. *The surface tension of the fluid measured.*—The observations so far described refer only to distilled water. The difference in the surface tension and density of various fluids will, of course, lead to the volume of a drop being different from that of water. Donald met this difficulty by introducing "drop-count factors" for various fluids likely to be required:

Water	1·0	Saline	1·0
Human serum	1·10	Cerebrospinal fluid	1·02
Rabbit serum	1·09	Peptone broth	1·2
Guinea-pig serum	1·06		

The actual volumes of these fluids delivered by a particular pipette are found by dividing the volume of water which the pipette would deliver by the factor for the fluid.

Donald arrived at these factors by dividing the number of drops of fluid per unit volume by the number of drops of water for the same volume.

For accurate work some such correction factor is of course essential, the error in volume amounting to 8 per cent. with some commonly used liquids.

We have re-investigated this matter by first dividing the weight of 10 drops of the liquid by the density, to obtain the volume. Then the conversion factor was calculated by dividing the volume of 10 drops of water (from the same pipette, and at the same rate) by the volume of 10 drops of the fluid used. The density was estimated by comparing the weight of a small vessel (capacity 0·8623 c.c.) when filled with water, and with the various fluids. Table IV gives the details of these observations.

It will be seen, for example, in the case of serum, that a pipette gauged to deliver 10 c.mm. water will deliver only $\frac{10}{1{\cdot}05} = 9{\cdot}5$ c.mm. horse-serum, 9·4 c.mm. guinea-pig serum or 9·7 c.mm. human serum. The errors involved in working without a conversion factor in these cases range from 3 to 6 per cent.

We are not prepared to say to what extent the conversion factor varies for different animals or for different individuals of the same species. Our results would suggest no variation in a species and but slight variation between species. On the other hand, our factors show some variations from those given by Donald.

TABLE IV.—*Estimation of Conversion Factors for Various Fluids.*

	Weight of 10 drops from Morse 53 at about 35.	Density.	Volume.	Conversion factor (approx.).
Water	0·2723 gm.	1	0·2723 c.c.	1·0
Saline (0·9 %)	0·2753 ,,	1·006	0·2736 ,,	1·0
0·45% carbolic in saline	0·2540 ,,	1·012	0·2509 ,,	1·08
Peptone broth	0·2339 ,,	1·004	0·2329 ,,	1·13
Human serum	0·2689 ,,	1·019	0·2638 ,,	1·03
	0·2713 ,,	1·023	0·2652 ,,	1·03
	0·2690 ,,	1·019	0·2639 ,,	1·03
	0·2711 ,,	1·022	0·2652 ,,	1·03
	0·2698 ,,	1·020	0·2645 ,,	1·03
Horse-serum	0·2623 ,,	1·020	0·2571 ,,	1·05
	0·2675 ,,	1·032	0·2593 ,,	1·05
Guinea-pig serum	0·2653 ,,	1·033	0·2568 ,,	1·06

There is no doubt that the necessity for a conversion factor limits the usefulness of measurement by drops, except for purposes in which an error of 5–8 per cent. does not matter.

For accurate purposes the worker must find the conversion factors himself for such materials as he habitually measures. The method used by Donald was to count the number of drops of water and of the liquid to be measured which would fill a certain volume, and this method is simple and sufficiently accurate if over 50 drops of water are counted.

It will be found that thin suspensions of bacteria and other particulate bodies have practically the same factor as the menstruum, and that dilutions of serum about 1 : 8 or higher differ immaterially from water.

In practice the conversion factor is applied in two ways: (*a*) When using, for instance, serum with a particular pipette, what is the volume per drop? Find the water volume from the nomogram and divide by the factor. (*b*) It being required to deliver a particular volume of serum, multiply this volume by the factor and then select a pipette which will deliver this water volume.

It will frequently happen that the pipette required falls between two Morse numbers; in such a case the pipette may be gauged in the two Morse holes and cut between the marks.

CONCLUSIONS.

The effect of the rate of delivering drops upon the weight of the drops is demonstrated, and the relation of the external diameter of the dropping-tube to the weight of the drop is shown over the whole range in which drops of water can be delivered in air.

Nomograms have been prepared to show at a glance the relationships between drop-weight, drop-rate and size of pipette.

For the practical measurement of small volumes of fluids to a high degree of exactness it is necessary to take special account of the accuracy—

(*a*) Of the gauges used for measuring the pipettes.

(*b*) Of the measurement so made.

(*c*) Of the measurement of the drop-rate.

(*d*) Of the estimation of the conversion factor for fluids of different surface tensions and densities.

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DIFFERENCES IN CELL CONTENT SHOWN BY STAINING WITH ACID AND ALKALINE SALTS OF CONGO: A METHOD OF DEMONSTRATING ELASTIC TISSUES.

T. H. C. BENIANS.

From the Pathological Department, Prince of Wales's General Hospital, Tottenham, London.

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A SOLUTION of Congo red, which is the alkaline salt of the dye, suffers a dramatic change of colour when the reaction of the solvent is made acid; the acid salt is formed and the solution goes a deep blue. This is equally true both as regards the ordinary organic and inorganic acids. The change is reversible.

If the solution of Congo is mixed with an equal part of blood-serum or egg albumen and acid is now added until the fluid has a strongly acid reaction, it is found that the dye is not blackened. The albumen, whether by combining with the acid or in some other way, exerts a protective influence on the dye, which remains as the red alkaline salt. If a solution of gum is used instead of albumen the dye is not protected from the acid and turns black.

It seemed probable that this protective character of the blood-serum might be shown by the cell content of certain of the body tissues, and that these tissues when stained with Congo would resist the blackening effect of the acid, whilst other non-protoplasmic tissues would not protect the Congo and would be blackened by the acid.

It was found that acids vary greatly in their power of blackening tissues stained with Congo red. Stained sections may be exposed to 50% acetic acid for hours without any tissues except the elastic being blackened, whereas with hydrochloric acid it is not safe to go beyond 0·25% to get a differential effect—0·5% of this acid blackens the whole section.

The object of the investigation then was to see which tissues contain a substance that would exert this protective influence.

Wet Preparations.

Method.—Tissues well fixed in formalin are cut on a freezing microtome, stained for 10 minutes in a 1% solution of Congo red, washed for a few moments in clean tap-water and treated with 5% acetic acid or 0·25% aqueous HCl. They are then examined wet in the acid solution under a cover-glass.

This method gives the clearest and most striking results. The elastic fibres, even the finest, are clearly and sharply picked out in a deep bluish-black colour. The other tissues in general are not affected by the acid.

Muscular tissues are stained a bright clear red ; fibrous tissues are also red, but with a tendency to be slightly browned.

Cutaneous epithelium shows a lack of staining of the superficial layers, a heavy ill-defined darkening in the region of the *stratum granulosum*, and a clear red in the deeper layers. Cutaneous and intestinal secreting glands stain a clear red although they do not stain deeply. Cartilage remains practically unstained, or just a faint red. Fibro-cartilage such as that in the epiglottis shows a remarkable network of elastic fibres with clear unstained tissue between.

The *ligamentum nuchæ* of the ox in longitudinal section shows a general blackening, in which the coarse fibres are clearly defined with a small amount of red interstitial tissue. On cross-section the appearance is quite different; each fibre shows as a ring of dark tissue with a clear centre, stained a pale red. It appears that the centre part of these fibres has the property of protecting the stain from acid whilst the periphery has not.

The colloid of thyroid sections stains red but does not blacken with acid.

Permanent Preparations.

These offer a good many difficulties, and their real permanency will be yet to prove. The first difficulty lies in the fact that in dehydrating the tissues the acid is removed from the elastic, which immediately becomes red again. If, however, the whole of the section is blackened by a mineral acid, it is found on differentiating with alcohol that the elastic retains the acid longer than the other tissues, although the finer elastic fibrils soon decolorize and the differentiation becomes a matter of degree. Even so good preparations may be made though they lack the sharpness and brightness of the wet section. Clearing is another unsatisfactory point, since Congo-stained sections do not seem to clear properly with xylol but always remains somewhat opaque; moreover, sections stained this way often shrink in the xylol.

Method.—Stain as described above with Congo red for 10 minutes. Wash. Treat with 1% picric acid for ½ minute or until the section has entirely changed colour. Pour off the picric acid and wash in acid alcohol for 3 or 4 minutes. The section is then differentiated and dehydrated by washing in absolute alcohol, changing the alcohol from time to time, until the bulk of the tissues are a dull red, a matter of a minute or two, observing the section occasionally under the microscope. Clear in xylol and mount.

Sections cut with a freezing microtome do fairly well by this method, but the results are not so good with paraffin sections. The process of clearing the tissues and infiltrating them with paraffin seems to denaturate the tissues very largely, so that their selective staining properties are not well shown.

A STUDY OF THE ROUS FOWL SARCOMA NO. 1: I. FILTERABILITY.

W. E. GYE AND C. H. ANDREWES.

From the Medical Research Council's Field Laboratories, Mill Hill.

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" . . . the findings with the chicken-tumours largely demolish the theoretical basis on which objections to an extrinsic cause for cancer have been built up " (Rous, Murphy and Tytler, 1912). The validity of this statement depends upon two assumptions: first, that new growths form one homogeneous group, and secondly, that the filterable fowl tumours are members of the group.

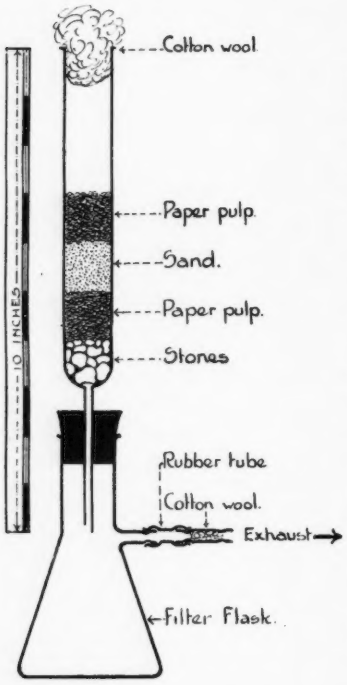
The first assumption is universally accepted and needs no comment. The second assumption has never been formally resisted; we know of no paper in which the evidence put forward by Rous and his collaborators has been tested and discussed, and in which an opposite conclusion has been reached. Nevertheless, it cannot yet be affirmed that the filterable fowl sarcomata are accepted as true tumours in the sense that the Jensen rat sarcoma or the Jensen mouse carcinoma are so accepted. The reason for this evident hesitation is simply this—that the fowl sarcomata are transmissible to other fowls by the inoculation of cell-free filtrates. If this latter fact were still unknown we do not doubt that the tumours would be accepted as typical highly malignant neoplasms.

It is of supreme importance, therefore, to study this property of filterability of the fowl tumours, to determine how far it is constant and to discover the conditions under which it varies.

METHODS OF FILTRATION.

A fowl bearing a large tumour is taken and killed with chloroform. The tumour is removed aseptically and healthy tumour-tissue separated from normal muscle and necrotic tissue. Sometimes this separation is impossible, as the tumour consists of a rather dry necrotic mass which replaces the whole of the pectoral muscles. But wherever possible, soft healthy tumour-tissue is taken. The tissue is minced very fine with scissors, and 2 c.c. of the mince is measured, by means of a Bashford syringe, into a large—15 cm. diameter—porcelain mortar. Silver sand, which has been washed in acid and distilled water and then sterilized, is added, and the tumour mince is then vigorously rubbed with a large heavy pestle (22 cm. long). It is important not to add fluid until the

tissue is completely disintegrated, which is indicated when small red particles are no longer visible. 200 c.c. of Ringer's fluid is now added in small quantities at a time, the mixture being stirred round with the pestle until an even, thin emulsion is obtained. The first step in filtration now is to remove the large particles of tissue *débris* and the sand. This is achieved by passing the thin emulsion through layers of paper-pulp and sand. The accompanying figure shows very clearly how the filter is made and a description is therefore unnecessary. The paper-pulp is bought in compressed blocks; it is shredded and then soaked in distilled water, in which it becomes soft and pliable. In making up the filter the paper-pulp is pressed, by means of a thick glass rod, into a fairly compact layer an inch thick; the sand adjusts itself. Through such a filter, properly made, the aqueous cream of tumour extract passes readily under gravity. The filtrate is transparent, and as a rule faintly yellow-red in colour. It contains no cells; that is, if such a filtrate be spun vigorously and the deposit examined microscopically no cells can be found.



To obtain 50 c.c. of such a filtrate requires about half an hour. As a rule such a large volume is not required, and since the first 20 c.c. of filtrate comes through much more rapidly than the second 20 c.c., it will be noted that this part of the process is not time-consuming.

The filter candles which we have used in the next stage are the Mandler candle and the Chamberland L series, which are graded L_1 (coarse), L_2, L_3 and L_5, the fineness increasing with the number. The Chamberland candles, which are of the Kitasato pattern, are used when small quantities, *e.g.* 10 c.c., of filtrate are required; the Mandler type when large quantities are sought.

The sand filtrate is now filtered through one or other of the above candles. An ordinary water-pump is used to produce the necessary pressure (20–30 mm. mercury). Filtration is very rapid. If it is slow the candle is discarded and another taken. The time required to obtain 10 c.c. of filtrate through an L_2 candle, measuring from the moment filtration through paper-pulp and sand is begun, is from 15–20 minutes.

The proportion of tumour emulsion to diluting fluid is very important. The figures given—1 gm. to 100 c.c.—have been found to be best. When the proportion of tissue is increased filtration is much slower and the final filtrate is not very effective. Using the quantities recommended, it is possible sometimes to obtain Mandler or L_2 filtrates which produce tumours in doses

as small as 0·01 c.c. As a rule, however, it is necessary to employ larger doses, 0·5–1 c.c.—*i. e.* the equivalent of 0·01–0·005 c.c. of tumour.

Such filtrates through L_2 and Mandler candles when examined with the dark field apparatus are almost optically empty; occasional particles are seen but their rarity is surprising. Needless to say, the ultramicroscope reveals immense numbers of particles.

THE DRYING OF TUMOUR-TISSUE.

The production of tumours with dry tumour-tissue is an important additional piece of evidence that it is unnecessary to inoculate the living cell in order to carry on a tumour in series.

Our method of drying tumour-tissue is as follows: Healthy tumour-tissue is minced very fine and spread out in a thin layer in a large Petri dish. The Petri dish is placed over P_2O_5 in a desiccator, which is then evacuated by means of a Geryk pump. The P_2O_5 liquefies in 5–6 hours and is then replaced. At room temperature the tissue is almost dry in 24 hours. The final stage of drying is completed in the cold room. The dry scales are powdered and stored in the cold room.

When used, 1 gm. of the dry powder is made into a thick emulsion with distilled water, and birds are inoculated with 0·5 c.c. by means of a Bashford syringe and needle.

The following general results are obtained when filtrates or dried tumour-tissue are inoculated into Plymouth Rock chickens:

Dried tissue injected in the dose mentioned causes a tumour to form which is palpable in 14 days, and which becomes large and kills the host generally within 5 weeks. There is a considerable irregularity in the result, however, and it is advisable to keep a number of samples of dried tissue as a reserve of material.

Filtrates through paper-pulp and sand are exceedingly virulent. Tumours can be induced with very small doses, *e. g.* 0·001 c.c.; the smaller the dose the longer time it takes to induce a tumour. With a dose of 0·2 c.c. a tumour usually appears in 7 days and kills the bird in 14–16 days.

Chamberland L_1 filtrates are also very virulent, but less so than paper-pulp sand filtrates. Although the L_1 candle is relatively coarse, it removes a large amount of infective material during filtration. It is difficult to obtain precise comparisons between filters because of the great variability in the properties of candles; one L_1 candle may differ from another very considerably both as to rate of filtration and to infectivity of filtrate. But generally speaking it may be said that 0·2 c.c. of paper-pulp sand filtrate is equal in infectivity to 1 c.c. of L_1 filtrate. In making such a test 0·2 c.c. paper-pulp sand filtrate is made up to 1 c.c. by addition of saline, and a half-grown bird is then inoculated in one breast with the diluted filtrate, in the other breast with 1 c.c. L_1 filtrate. Thus it may be said that on an average of a large number of tests of this kind the L_1 candle removes four-fifths of the infective material from a sample of sand filtrate.

When L_1 filtrate is passed through an L_2 candle there is a further loss of infectivity.

Although L_2 filtrates are almost always effective, the dose required to produce a tumour is generally 0·5–1 c.c.

L_3 candle filtrates are rarely infective in doses of less than 1 c.c.

L_5 candle filtrates are innocuous.

Mandler candle filtrates are almost as infective as L_1 filtrates.

It is evident from this brief statement that filter-candles absorb a large part of the agent—or agents—which cause the fowl sarcoma. The results obtained with different tumour filtrates are not, however, regular. Filtrates from some tumours are exceedingly virulent, from others almost innocuous; that is to say, that 1 c.c. of an L_2 filtrate of one tumour is sometimes as effective in inducing the formation of a tumour as is 1 c.c. L_1 from another tumour. These differences were attributed for a long time to inevitable variations in biological experiments, and to differences in filter-candles, choice of tumour material and so forth.

The following series of a tumour, in which absolute differences were exhibited, shows beyond dispute that the fowl sarcoma itself varies in its filterability.

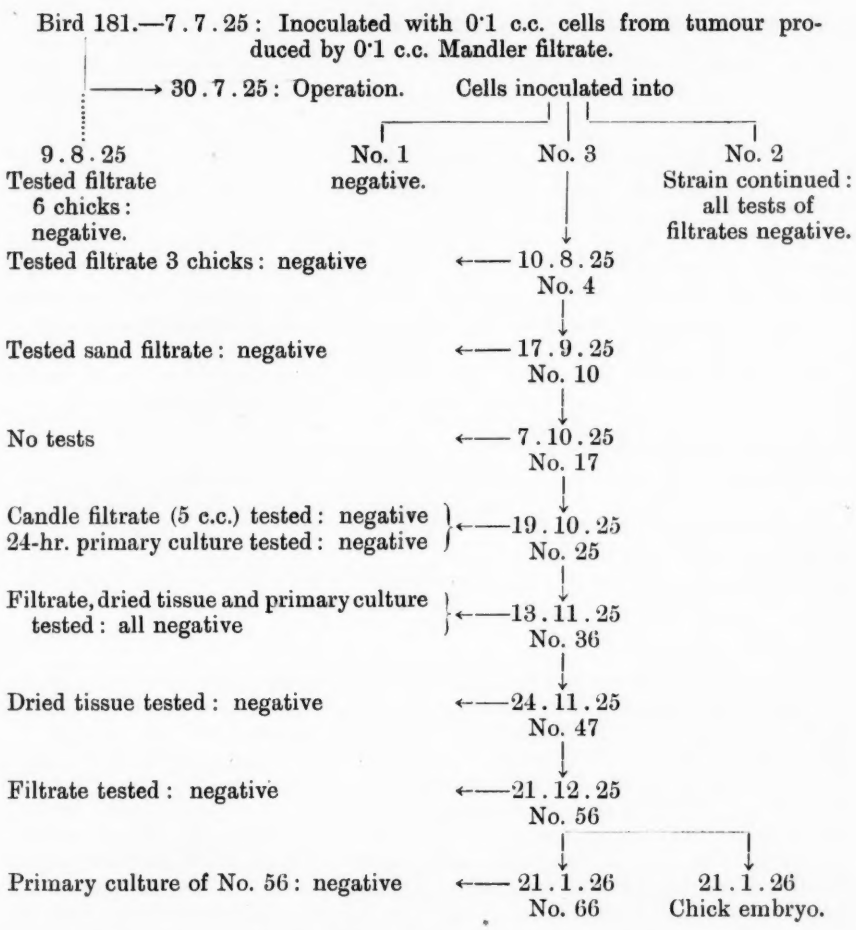
On the 7th of July, 1925, a tumour which had been produced by the inoculation of 0·1 c.c. of Mandler filtrate a month before was taken and minced with scissors. A half-grown Plymouth Rock bird, No. 181, was inoculated in the right breast with 0·1 c.c. of the cell emulsion. A tumour was produced which grew very slowly. On the 30th of July, 23 days later, an operation was performed, the tumour being incised and a portion of the growth removed. Three chickens, Nos. 1, 2 and 3, were inoculated with the tumour thus removed. The tumour fragments left in Bird 181 grew rapidly after the operation, and the bird was killed on the 9th of August. Six chickens were inoculated with a Mandler filtrate of the tumour and all remained negative.

No. 1 chicken of the new series did not develop a tumour.

No. 2 developed a tumour which was tested as to its filterability, which was negative, and was transplanted for several generations. Eventually it was abandoned.

The tumour which grew in chicken No. 3 was carried on as a routine strain—by the inoculation of cells at each passage—as is indicated in the accompanying table (I). It will be observed from this table that the tumour in each generation—except one—was tested as to its powers of inducing tumour-formation with cell-free filtrates or with dried tumour-tissue, and that for a period of six months all such tests were absolutely negative. Thus the tumour had become non-filterable. During this period the histological structure of the tumour remained constant, except that the bundles of spindle-cells were more compact. Metastasis formation was infrequent, and when metastases did occur—in the liver, for example—they consisted of firm white discrete nodules. The characteristic hæmorrhagic points and masses so frequently observed in metastases of this tumour, especially in the liver and spleen, were entirely absent during this period. Further, the tumour lost that stupendous power of proliferation which is so noticeable in the filterable stain: it became relatively benign. Moreover it showed fluctuations in growth energy which are characteristic of mammalian transplantable tumours.

TABLE I.


Bird 181.—7 . 7 . 25 : Inoculated with 0·1 c.c. cells from tumour produced by 0·1 c.c. Mandler filtrate.
30 . 7 . 25 : Operation.
Cells inoculated into
9 . 8 . 25
Tested filtrate 6 chicks : negative.
No. 1
negative.
No. 3
No. 2
Strain continued : all tests of filtrates negative.
Tested filtrate 3 chicks : negative
10 . 8 . 25
No. 4
Tested sand filtrate : negative
17 . 9 . 25
No. 10
No tests
7 . 10 . 25
No. 17
Candle filtrate (5 c.c.) tested : negative
24-hr. primary culture tested : negative
19 . 10 . 25
No. 25
Filtrate, dried tissue and primary culture tested : all negative
13 . 11 . 25
No. 36
Dried tissue tested : negative
24 . 11 . 25
No. 47
Filtrate tested : negative
21 . 12 . 25
No. 56
Primary culture of No. 56 : negative
21 . 1 . 26
No. 66
21 . 1 . 26
Chick embryo.

It will be observed from Table I that cells from tumour 56, which had grown slowly, and was firm in consistence, were inoculated into Bird No. 66 as a routine inoculation, and also into a chick embryo by Murphy's method. This latter transplantation, which was carried out for us by Dr. A. M. Begg, to whom our thanks are due, was done as an attempt to restore to the tumour its lost property of filterability. It was hoped that a temporary sojourn in an embryo, in which resistance to foreign cells is absent, would enable the tumour-cells to re-acquire their proliferative energies. Table II shows the result of this experiment and the subsequent behaviour in the normal routine transplantation.

TABLE II.

21.12.25: 0·1 c.c. cells from No. 47 inoculated into Bird No. 56

21.1.26: Cells inoculated in chick embryo	21.1.26 No. 66	
	3.2.26 No. 72	→ No. 66 tested L_1 filtrate: chicken died of intercurrent disease.
Tumour from embryo inoculated 1.2.26 (11 days) in No. 70	20.2.26 No. 77	→ No. 72 tested 1 c.c. Mandler filtrate: positive.
10.3.26 (37 days) No. 86	8.3.26 No. 80	→ No. 77 tested: L_1, L_2, L_5 filtrates. (L_1 and L_2 positive, death in 17 days; L_5 negative.)
	18.3.26 No. 88	

No. 70 primary culture tested: Negative 28.3.26

On the 1st of February, 1926, Bird 70 was inoculated with 0·2 c.c. of the tumour which had grown in the chick embryo. A tumour developed slowly, and became palpable as a firm lump the size of a hazel-nut on the 19th of February. The bird, though not ill, was killed on the 10th of March (37 days after the inoculation). "The tumour was the size of a walnut, hard and yellow, with necrotic areas in the centre. The tumour had permeated through the body-wall to the peritoneal cavity, leading to extensive adhesions between the right lobe of the liver, the gall-bladder and the intestines, the growth binding these organs together being hard and fibrous. One metastasis was found in the right lung, none in the left, one on the pericardium, and one 'pin-point' in the liver; none elsewhere." This is quoted from the protocol because it is typical of the slow-growing strain. The test as to filterability of this tumour is negative at the time of writing.

With regard to the transplantation of tumour-cells from 56 to 66, it will be noted that No. 66 was killed on the 3rd February, 13 days after inoculation. The protocol notes are as follows: "3 Feb./26: Bird ill; blood-stained fluid issuing from mouth. Killed. *Post mortem*: Tumour 7 cm. × 4·5 cm., very necrotic. Thorax full of blood-stained fluid containing necrotic *débris*. A clot of blood on right lobe of liver. Metastases in lungs." Thus the tumour had in this bird altered its character. It was transferred to No. 72.

The test of filterability failed by reason of the death from intercurrent disease of the chicken tested.

The tumour which grew in No. 72 was a typical filterable Rous sarcoma: a large soft hæmorrhagic tumour, with internal hæmorrhagic metastases. The filtrate—1 c.c. Mandler filtrate—of this tumour killed a bird in 15 days. Filterability had therefore returned.

It may be mentioned that while the above experiments were in progress another, readily filterable, strain of the tumour, recently obtained from Dr. Rous, was being carried on: this seems to exclude the possibility that our results could be explained by any variations in our technique.

Thus the property of filterability is variable; it is not fixed and unalterable like the microscopic anatomy of the tumour. It may be for long periods of time entirely absent as judged by our present somewhat crude tests. We may definitely conclude that it may vary from a very low to a very high level.

The explanation of this sudden disappearance and reappearance of filterability has not yet been definitely determined. If the essential cause of the tumour is a filterable virus—as one of us has urged (Gye, 1925)—are we to assume that the virus is sometimes absent? If that were so, then the essential cause is not always present, which is self-contradictory, since by essential cause we mean an invariable factor. This aspect of the tumour will be discussed in a subsequent paper.

With regard to the fundamental question—Is the Rous tumour a true new growth?—one comment only is needed: it is inadmissible to argue that filterability constitutes a real difference between the Rous fowl sarcoma and other transplantable tumours.

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SELF-CONTROL AND INHIBITION IN THE ADRENAL GLAND.

W. CRAMER.

From the Laboratories of the Imperial Cancer Research Fund, London.

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THE secretory activity of the adrenal gland is under the control of the sympathetic nervous system: stimulation of the splanchnic nerves induces a secretion of adrenalin. Since adrenalin itself stimulates sympathetic nerve-endings, it would follow that the injection of adrenalin itself should elicit a further secretion of adrenalin from the gland. It would appear therefore that if once a sufficiently massive secretion of adrenalin had been induced, the activity of the gland should continue to stimulate itself to further activity until it is exhausted, a short stimulation of the gland initiating a kind of avalanche phenomenon. We know that this is not so, since the effect of an injection of adrenalin passes off with extreme rapidity. There must exist, therefore, a controlling mechanism which protects the gland against such self-stimulation. This mechanism is disclosed by a study of the appearance of the gland, by means of the osmic vapour method, after an injection of adrenalin.

Before discussing the effect produced in this way we must briefly recall the appearance of the normal resting gland of a mouse as shown by this method and described in a previous paper (Cramer, 1919). It may be added that in all our observations the whole gland has been cut in serial sections, so that it is possible to make a comparison between corresponding parts of different glands. This precaution is essential. The following descriptions refer to appearances in section through the control part of the medulla, either along its short or its long axis, but nearer that pole from which the central vein eventually emerges. The appearance of the normal gland is illustrated in Fig. 1. The medulla is large in relation to the cortex. The bulk of the latter is made up of columns of cells of the zona fasciculata, while the innermost zone—the zona reticularis—surrounds the medulla as a sharply defined continuous, even, narrow band. The lipoid globules are situated mainly in the cells of the outer two-thirds of the cortex. When as a result of exposure to cold or of an injection of β-tetrahydronaphthylamin the gland is actively

secreting adrenalin, the secretion begins and is most massive in the cells situated in the centre of the medulla around the central vein and its tributaries. At the same time the cortex shows signs of activity by a spreading of the lipoid globules from the periphery towards the central parts, so that they are distributed over the whole cortex. As has been pointed out in a previous paper (Cramer, 1919), the secretion of adrenalin by the medulla stimulates at once the formation of new adrenalin, and in this the innermost part of the cortex appears to play an important part. For if active secretion is prolonged, globules of materials having the same staining reaction as adrenalin appear in the innermost part of the cortex in the cells of the zona reticularis, which, like the active medulla, shows congestion. The occurrence of adrenalin—or a precursor—in the innermost part of the cortex has been confirmed by Hartman (1923). The point of importance in connection with the following observations is that the cortex participates in the activity of the medulla, and that there is a particularly intimate close functional relationship between the medulla and the immediately adjacent cells of the cortex in the zona reticularis.

After an injection of adrenalin, the peripheral cells of the medulla—those immediately adjacent to the cortex—lose their adrenalin (see Fig. 2). They thereby lose the typical appearance of medullary cells, and may be mistaken for cells of the cortex. At first sight this gives the impression as if the zona reticularis had hypertrophied enormously at the expense of the medulla, which appears much smaller than in the normal animal. It is obvious that in the few minutes which have elapsed since the injection of adrenalin no such hypertrophy can possibly have taken place, and closer inspection reveals in this broad zone islets of typical medullary cells still filled with granules of adrenalin. Fig. 3 gives a high-power view of this zone. In some parts these groups of cells are still connected with the medulla and project like peninsulas into this zone. This broad zone therefore belongs to the medulla, and is composed of medullary cells which have lost their content of adrenalin in varying degrees. An adrenalin-free zone of the medulla has thus been interposed between the cells of the zona reticularis of the cortex and the adrenalin-containing cells of the medulla as the result of the injection of adrenalin. The formation of such a zone is interpreted as interfering with the interaction of cortex and medulla, which is essential for the normal functional activity of the gland. It is the morphological manifestation of the check which the gland imposes upon itself in this condition, and by which it protects itself against self-exhaustion. We propose to call this phenomenon "self control" of the adrenal.

The same phenomenon has been observed in another condition, namely, after thyroid feeding over a prolonged period. It was, in fact, first observed several years ago in mice fed with small doses of thyroid over three months, and was then recorded in the accompanying drawings (Fig. 4). The adrenalin-free peripheral zone, with occasional islets of cells containing sparse granules of adrenalin, is particularly distinct in this condition. It was possible to demonstrate conclusively that this zone belonged to the medulla, and not to the cortex, by discontinuing the thyroid feeding and noticing the behaviour of this zone during the recovery. Fig. 5 is taken from the adrenal of such an animal when after $3\frac{1}{2}$ months of thyroid feeding the administration of thyroid

had been discontinued for three days, and shows the gradual reappearance of adrenalin granules in the cells of this zone. The gland still shows the broad zone of adrenalin-free medullary cells, but the islets of cells containing adrenalin have become much more numerous. Fig. 6 gives a high-power view of this zone, showing here and there cells or cell-groups renewing their load of adrenalin. In some of these cell-groups the granules of adrenalin are exceptionally large.

At the time when these observations on thyroid-fed animals were first made the significance of this process was not clearly understood. Its occurrence after injection of adrenalin has enabled us to interpret it, as stated above, as a mechanism of " self-control " of the gland.

In many of the thyroid-fed animals the cortex also exhibits a change in the distribution of the lipoid globules. Instead of being situated mainly in the outer half or two-thirds of the cortex—that is, the zona glomerulosa and the outer half of the zona fasciculata, as they do in the normal resting gland—they remain in the cells of the zona glomerulosa but disappear from the outer part of the zona fasciculata. Sometimes the innermost cells of the zona fasciculata are filled with these globules, so that a narrow band of lipoid-containing cells surrounds the medulla. But in other cases there is an extensive disappearance of the lipoid from the cortex, except, as already stated, the narrow peripheral zone of the cells of the zona glomerulosa. This cortical change frequently accompanies the medullary change described above as " self-control," but does not always do so and therefore has a different significance. The key to its interpretation is furnished by the following observations, which show that it is the outstanding feature of the change induced in animals exposed to heat. As will be shown, it represents an inhibition of the functional activity of the gland.

It is necessary to recall here briefly the conception which we have developed in previous papers of the thyroid-adrenal apparatus as a factor in the heat-regulation of the body (Cramer 1916, 1918, 1919, 1920 and 1924). We showed that the active secretion of the hormones of these two glands increases heat-production. Adrenalin by its peripheral vaso-constriction also diminishes heat-loss. It is clear, therefore, that these two hormones affect both the physical and the chemical heat-regulation of the body. Exposure to cold, which calls for increased heat-production and diminished heat-loss, is a powerful stimulus to the functional activity of these glands. Conversely, prolonged functional activity of these glands not due to stimulation by a cold environment produces fever accompanied by rigor. This conception has been confirmed by the work of Boothby and Sandiford (1923), who measured the calorigenic action of adrenalin, by Hartman (1923) and by Cannon, who both confirmed by different methods that cold is a powerful stimulus to the adrenal gland. It is clear that the regulation of the body-temperature through increased functional activity of the thyroid-adrenal apparatus can only be efficient in one direction—namely, in the direction of maintaining the body-temperature at a higher level than that of the surrounding environment. If, now, an animal such as the mouse or the rat, which have no sweat-glands andc annot increase heat-loss through perspiration, is placed in a warm environment having a temperature approaching that of the animal itself, regulation by heat-loss is eliminated. Then even a slight

activity of the thyroid-adrenal apparatus would, by increasing heat-production, necessarily lead to a hyperpyrexia and endanger the life of the animal. It would, in fact, produce heatstroke. Experimentally this condition can be reproduced by the injection of β-tetrahydronaphthylamin into animals kept in different thermal environments. We have shown in a previous paper (1920) that a slight stimulation of the thyroid-adrenal apparatus by the injection of a dose of β-tetrahydronaphthylamin, so small that it produces not even a rise in temperature if the animal is kept in a cool environment, will produce hyperpyrexial heatstroke if the animals are kept in a moderately warm room, or even if several animals are crowded together in a small cage. If in a hot environment adrenal activity is likely to endanger the life of the organism, we may expect that the organism will react to exposure to heat by an inhibition of the adrenal gland.

Mice are very sensitive to heat. It is possible to keep mice alive for weeks at a temperature of 37°–38° C. The animals remain quite well. But even a slight increase in the temperature beyond 38° C. will kill them, although the external temperature may only be one or two degrees Centigrade above their body temperature. When one examines the adrenals of mice kept for several days at 37°–38° C. by the osmic vapour method the medulla frequently shows no obvious change. The cells are fully loaded with adrenalin granules. Some animals may show the phenomenon of "self-control" just described in which adrenalin disappears from the peripheral cells of the medulla. This change, however, is not always present. But the cortex always shows a distinct change, namely an extensive disappearance of the lipoid globules. These disappear completely from the zona fasciculata in which they are normally present, and only a narrow band of lipoid-containing cells remains in the outermost layer of the zona glomerulosa. At the same time the animals are in an excellent condition of nutrition and show abundant adipose tissue. The glandular adipose tissue in which the adrenal lies embedded is filled with ordinary fat, with the result that the normal brown colour has disappeared and it looks yellow, like ordinary adipose tissue. The disappearance of lipoid from the cortex is therefore in striking contrast to the condition in the other deposits of adipose tissue. The statement made above that the adrenals of mice exposed to heat show sometimes very distinctly the phenomenon of "self-control"—disappearance of adrenalin from the periphery of the medulla—is illustrated in Fig. 7. In such cases there is also a disappearance of lipoid from the cortex, but this is not as complete as in animals in which the medulla has retained its normal load.

It is known that the presence of lipoid in the cortex is absolutely independent of the nutrition of the animal. For even in complete emaciation the cortex has its normal load of lipoid. The fact that in starvation the cortex retains so tenaciously its lipoid when the insistent demand exhausts all the other deposits of fatty material gives a special significance to the disappearance of lipoid as a result of exposure to heat. Such a complete disappearance of lipoid has been observed so far only in severe pathological conditions leading to the death of the organism—for instance in many severe bacterial infections.

It will be recalled that activity of the gland, such as can be produced, for instance, by exposure to cold, is associated with a spreading of the lipoid from the outer half over the whole cortex—a migration from the periphery of the

cortex towards the medulla. Exposure to heat produces a disappearance of the cortical lipoid. We conclude, therefore, that *activity of the gland is accompanied by a spreading of the cortical lipoid over the whole cortex, inhibition of the gland by a disappearance of the cortical lipoid.*

SELF-CONTROL AND INHIBITION IN PATHOLOGICAL CONDITIONS.

The existence of these two mechanisms of "self-control" and "inhibition" throws light on some pathological problems of the adrenal. Since the mechanism of "self-control" comes into action by thyroid feeding, *i. e.* in experimental hyperthyroidism, it is likely that the condition of this mechanism may play an important part in clinical hyperthyroidism. Inefficiency of this mechanism may be as important an ætiological factor in Graves's disease as the degree of hyperfunction of the thyroid. A degree of thyroid hyperactivity which in a normal organism may produce a condition remaining within physiological limits owing to the efficiency of the mechanism of self-control in the adrenal, will produce a greatly exaggerated effect if this mechanism is not efficient. From this point of view the functional condition of the suprarenal may be an important factor in the ætiology of Graves's disease. The suggestion that the adrenals become involved in Graves's disease has also been made on clinical grounds by Langdon Brown (1923). He points out that the disordered function of the adrenals may become the predominant factor in the course of the disease, and may carry it on, so to speak, even when the hyperactivity of the thyroid gland has subsided. It is not suggested here that this occurs in every case of the disease. It is well known that different cases of Graves's disease react very differently to similar treatment—a fact which suggests differences in the underlying condition in different cases. There is also the curiously paradoxical fact that in some cases of Graves's disease adrenalin given repeatedly by the mouth greatly relieves the condition. The effect is puzzling, and contrary to all *à priori* expectation. The demonstration of the mechanism of self-control in the adrenal which can be brought into action by adrenalin offers the solution of this therapeutic puzzle. The same explanation may perhaps apply to the beneficial effects which have been obtained quite recently by the oral administration of iodine (Plummer 1923, Starr 1924, Fraser 1925). This produces in many cases a remission which is as abrupt and as extensive as that following subtotal thyroidectomy. The effect is, however, not permanent, and the disease cannot be cured in this way. If the administration of iodine is stopped, the metabolic rate rises again rapidly and the symptoms reappear in a few weeks. It is interesting to note that this therapeutic puzzle was discovered as a result of a mistake in 1863 by Trousseau, who, by a lapse of the pen, prescribed to a patient suffering from Graves's disease tincture of iodine instead of tincture of digitalis.

The disappearance of the cortical lipoid in response to physiological conditions imposing an inhibition on the activity of the gland gives a clue to the interpretation of this phenomenon under pathological conditions. As already stated, in human post-mortem material it has been observed frequently though not constantly in many virulent bacterial infections. In a previous paper

(Cramer 1919) we have recorded it as a terminal stage in such experimental infections as gas gangrene, streptococcal septicæmia and diphtheria. In these conditions there is in addition a depletion of the load of adrenalin from the adrenal medulla. In a separate paper we intend to record observations on the intermediate stages of bacterial infections, which show that many bacterial toxins stimulate the activity of the gland. Only if the infection is of sufficient virulence does the inhibitory effect manifest itself on the cortex, thus preventing it from carrying out its normal function of assisting the active medulla in recharging itself with adrenalin. The relation of the gland to bacterial infections will be discussed more fully in that paper.

SUMMARY.

The adrenal gland possesses the power to control its functional activity in such a way as to prevent the gland from stimulating itself to complete exhaustion. The existence of such a mechanism is necessitated by the fact that the gland is stimulated to secretion by the sympathetic, and secretes as its specific hormone a substance—adrenalin—which itself specifically stimulates the sympathetic. It is shown that the injection of adrenalin and also prolonged feeding with thyroid gland produce characteristic changes in the adrenal medulla, which effect a "self-control" of the gland.

In addition there is a mechanism which effects an "inhibition" of the functional activity of the gland. This mechanism is located in the cortex and manifests itself as a disappearance of the cortical lipoid. It can be most readily demonstrated by exposing an animal to conditions such as a hot environment under which an activity of the gland would lead to the death of the animal by heatstroke.

The bearing of these conceptions on the pathology of the adrenal and thyroid is briefly discussed.

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DESCRIPTION OF PLATES.

FIG. 1.—Adrenal of normal mouse. × 60. (Reprinted, by permission, from the *Sixth Scientific Report of the Imperial Cancer Research Fund.*) The lipoid granules have been dissolved out by the Canada balsam, and are represented by vacuoles. Note the sharp and continuous divisions between medulla and cortex.

FIG. 2.—Adrenal of mouse 20 minutes after injection of 0·015 mgm. adrenalin. × 66. Shows appearance of adrenalin from peripheral parts of the medulla—"self-control." The right-hand side of the dotted square still lies inside the medulla.

FIG. 3.—Same animal. High-power view of the area enclosed in dotted square shown in Fig. 2. × 740.

FIG. 4.—Adrenal of mouse fed for 3 months with small doses of thyroid gland. × 75. Shows essentially the same change as found after adrenalin. Gland has been cut through its short axis.

FIG. 5.—Adrenal of mouse fed for 3 months with small doses of thyroid gland. Thyroid feeding discontinued 3 days before mouse was killed. × 75. Appearance similar to that in Fig. 4, but numerous islets of adrenalin containing cells are beginning to appear in the peripheral part of the medulla, of which the bulk is still free from adrenalin. Gland cut along its long axis.

FIG. 6.—Same animal. High-power view of the area in the peripheral part of the medulla outlined in Fig. 5. × 460. Shows the gradual reappearance of adrenalin in isolated cells and groups of cells. There is a great difference in the size of the adrenalin granules, some of which are very coarse.

FIG. 7.—Atypical effect of heat. Adrenal of mouse kept for 2 days at 37° C. × 75. The medulla shows a disappearance of adrenalin from the peripheral parts similar to the change observed after adrenalin and thyroid feeding. There is also a patchy disappearance of the lipoid from the cortex, indicated in the figure by differences in shading.

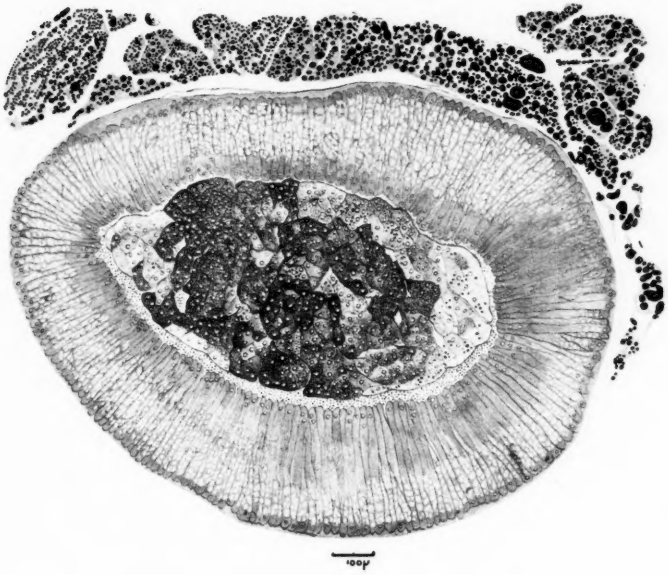

FIG. 1.

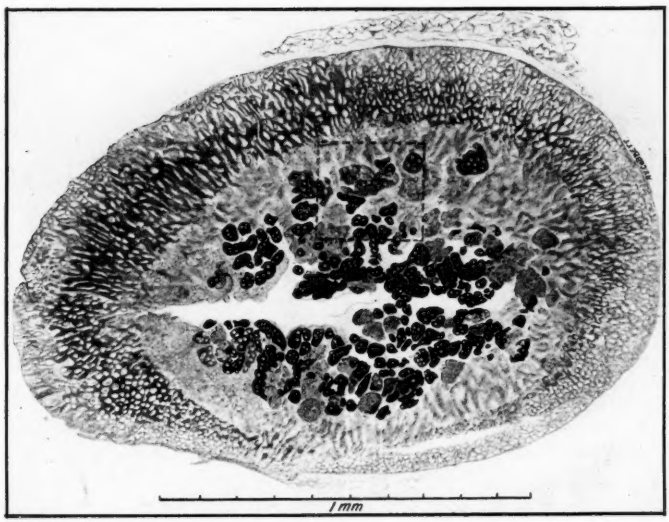

FIG. 2.

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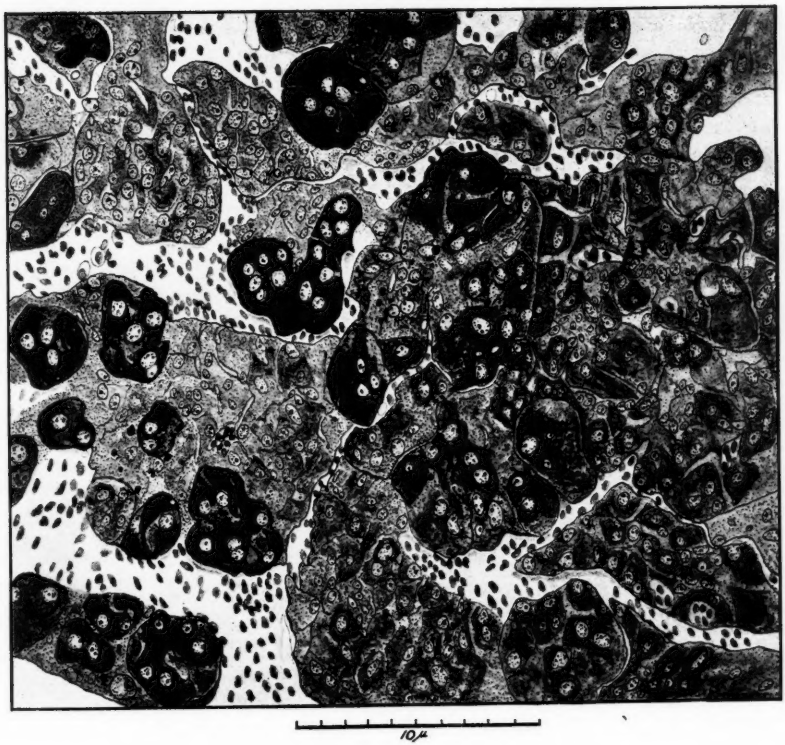

FIG. 3.

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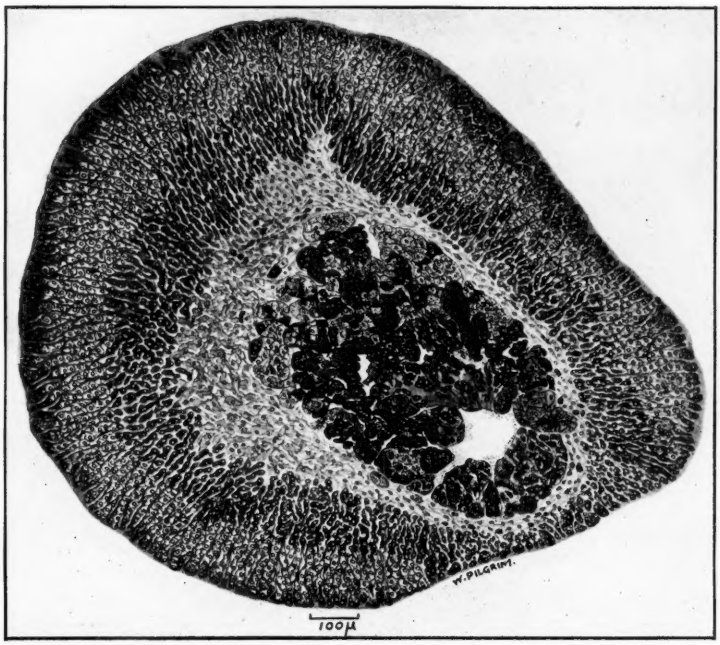

FIG. 4.

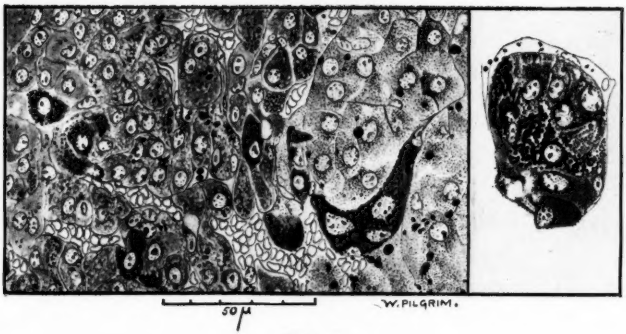

FIG. 6.

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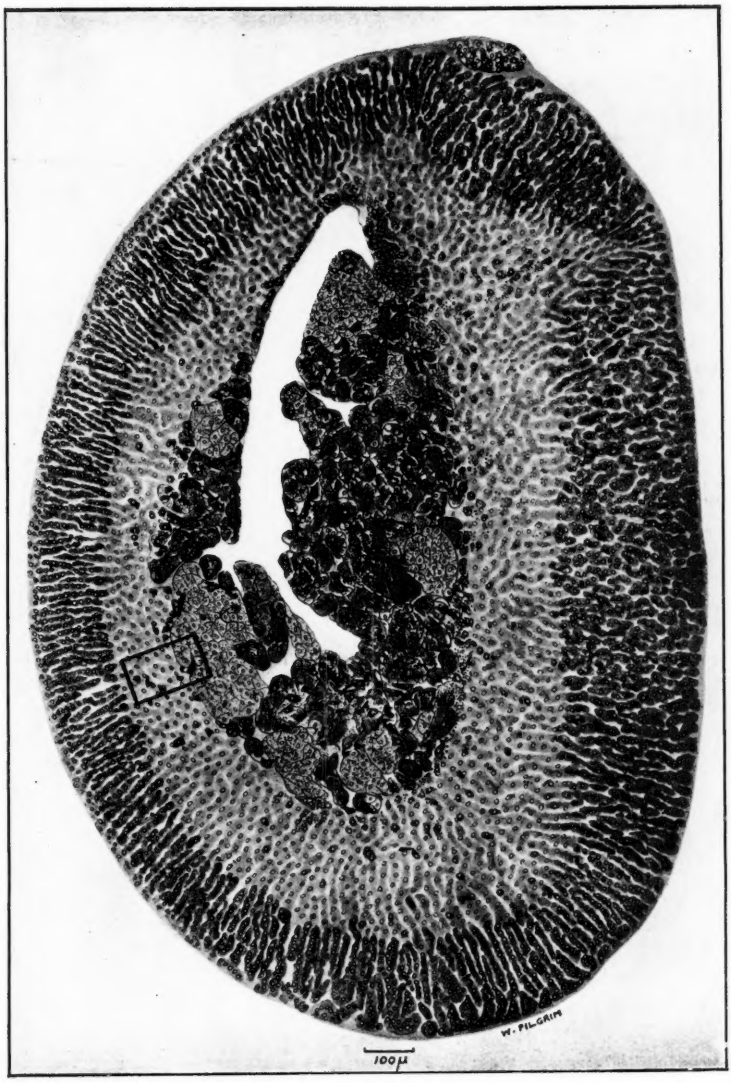

FIG. 5.

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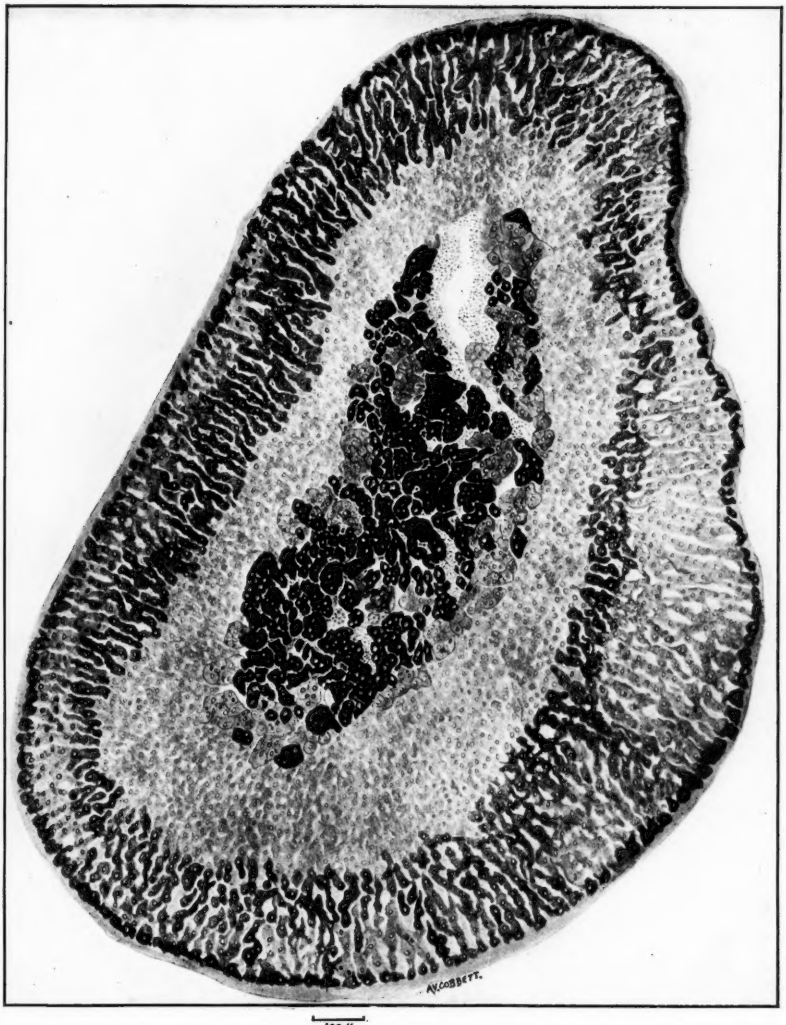

FIG. 7.